RAPID EVOLUTION IN AGROECOSYSTEMS: TRANSPOSABLE ELEMENTS AND EPIGENETICS IN THE COLORADO POTATO BEETLE

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ABSTRACT

Within agricultural ecosystems, humans and insects enter into complex relationships. Humans consider many of these insects to be pests, and exert significant pressures upon them, such as efforts to kill them using insecticides. One of the ways insects respond to these efforts is by rapidly evolving resistance to insecticides - but how they do this is not fully understood. DNA methylation, an epigenetic mechanism, and transposable elements, which are mobile genetic elements within genomes, may each play a role in shaping the way insects rapidly evolve in response to exposure to insecticides. Understanding the role of transposable elements and DNA methylation in the evolution of insects who live within agroecosystems can cast light on fundamental mechanisms of evolution while informing how we might live in better relation with these species.

These four chapters together provide support for complex interactions between insecticide exposure, transposable element activity, epigenetic inheritance, and adaptation to human-dominated agricultural landscapes in insects. First, I provide an overview of how insecticide-induced epigenetic effects can be inherited and may drive the evolution of resistance via epigenetic processes, contributing to ecological success in agroecosystems. Next, I utilize a large dataset of reports of insecticide resistance to determine if insect species evolve at different rates using survival analysis methodology. I then explore the diversity of transposable elements found within different populations of the Colorado Potato Beetle, *Leptinotarsa decemlineata,* to determine if these genetic elements play a role in the evolution of traits associated with living in agroecosystems. Finally, I analyze how DNA methylation in the Colorado Potato Beetle may be affected by exposure to insecticide, and if these changes to DNA methylation patterns are heritable and associated with genes known to be involved in insecticide resistance.

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CHAPTER 1: INTRODUCTION

This dissertation focuses on understanding the different aspects of an evolutionary pathway which may play a role in the rapid evolution of insecticide resistance in agroecosystems. Described more fully in Chapter 2, this pathway links exposure to insecticides, DNA methylation, transposable elements, and insecticide resistance. While researching this dissertation, evidence suggested that a pathway may exist in which transposable elements and epigenetics interact to facilitate the evolution of resistance to insecticide. The details of this possible pathway are detailed in the following chapters, but in brief, it appears that exposure to stress, including exposure to insecticides, can cause changes in epigenetic patterns, which can, either directly or through the activation of transposable elements, lead to heritable changes which contribute success in adapting to life in agroecosystems. Understanding this pathway may be able to shed light onto how some insect species are able to thrive in agroecosystems, while others are not able to do so. Each step in this pathway is supported by limited evidence, brought together from different species and experiments. The goal of this dissertation is to provide evidence for each step independently in the same species, in the hopes of providing enough information and support so that a comprehensive study which links all the parts together could be undertaken - and perhaps more importantly, funded.

These four chapters together provide support for complex interactions between insecticide exposure, transposable element activity, epigenetic inheritance, and adaptation to human-dominated agricultural landscapes in insects. The first two of the following chapters aim to answer this question in general, while the final two focus on a single

species, the Colorado potato beetle, *Leptinotarsa decemlineata*, a peanut-sized, yellow and black striped beetle that feeds primarily on the foliage of the potato plant (*Solanum tuberosum)*, a species domesticated in South America and now a member of agroecosystems around the world. The first paper outlines the framework for the following three papers, and the second uses a large-scale dataset to determine if insect species evolve at different rates. In the third chapter, I compare transposable elements between populations of this beetle to determine if long-term interaction with humans (and exposure to insecticides) has changed the composition of transposable element in their genomes. And in the fourth chapter, I assess how exposure the insecticide imidacloprid impacts DNA methylation, an epigenetic mark, in the Colorado potato beetle.

Chapter 1, *Transgenerational effects of insecticides — implications for rapid pest evolution in agroecosystems*, lays out the overarching framework and provides background for the primary questions this dissertation investigates. The aim of this paper is to synthesize a number of clues found in a broad range of fields which suggested that there might be a pathway which leads from exposure to environmental stressors, to changes in epigenetic marks and transposable elements, to heritable change which contributes to ecological success in agroecosystems. This paper is framed to answer these questions in light of the 'insecticide treadmill' as an example of heavy selective pressure.

Chapter 2, *Pesticide durability and the evolution of resistance: A novel application of survival analysis*, was an opportunity to use a large dataset and a conceptually relevant statistical model, survival analysis, in a new system. As part of the background for the following two chapters, we assumed that insect species evolve at different rates? However, we were unable to find a comparative study which could

provide evidence to back this up, and so we needed to do this assessment. We used data from the Arthropod Pesticide Resistance Database to look at the 20 species with the highest number of 'reports of resistance' to determine if the 'durability' (how long a pesticide remains effective) differs between these species. To do this, we adapted survival analysis methodology, which is generally used to determine how long medical patients 'survive' when given different treatments. In this study, the medical patients are the pesticides, and the different treatments are the arthropod species - so basically, the pesticides would "survive" longer when applied to arthropods who evolved more slowly - and pesticides should survive for a shorter time when encountering rapidly evolving pests who prove more robust - or evolve quicker. While we were not able to disentangle all the different reasons why insect species might evolve at different rates, we did find that there were differences between species. This meant that the following chapters, which were very much in progress, need not be in vain, since their role is to try and narrow down some of the reasons *why* different insect species might be evolving more rapidly than others.

In Chapter 3, *Transposable elements differ between geographic populations of the Colorado potato beetle, Leptinotarsa decemlineata***,** we explore the diversity of transposable elements found within different populations of the Colorado Potato Beetle to determine if these genetic elements play a role in the evolution of traits associated with living in agroecosystems, such as host plant preference and resistance to insecticides. We also examined differences in the number and diversity of transposable elements between populations of the beetle throughout North America, to determine if beetles living in what is now called the United States have more transposable elements than beetles living in

what is now called Mexico, which may indicate a role of transposable elements in the evolution of traits associated with living in agroecosystems.

Chapter 4, *Imidacloprid exposure affects transgenerationally inherited DNA methylation in the Colorado potato beetle, Leptinotarsa decemlineata,* was the first chapter conceived and the last to be completed. Here we attempt to better understand how DNA methylation may be affected by exposure to a pesticide, in this case imidacloprid, a common neonicotinoid, and if any changes to DNA methylation patterns can be heritable. This chapter takes both a zoomed-out approach, looking to see if exposure to insecticides trigger global changes in DNA methylation, and a zoomed-in approach, to see if specific sites of DNA methylation changes are associated with genes involved in stress reaction or insecticide resistance. We also examine possible interactions between methylation and transposable elements.

In the conclusion, I discuss the implications of this work in a broad sense, as well as look towards future directions this work could take in understanding the complexities of evolution within agroecosystems.

CHAPTER 2: TRANSGENERATIONAL EFFECTS OF INSECTICIDES — IMPLICATIONS FOR RAPID PEST EVOLUTION IN AGROECOSYSTEMS

1.1. Abstract

Although pesticides are a major selective force in driving the evolution of insect pests, the evolutionary processes that give rise to insecticide resistance remain poorly understood. Insecticide resistance has been widely observed to increase with frequent and intense insecticide exposure, but can be lost following the relaxation of insecticide use. One possible but rarely explored explanation is that insecticide resistance may be associated with epigenetic modifications, which influence the patterning of gene expression without changing underlying DNA sequence. Epigenetic modifications such as DNA methylation, histone modifications, and small RNAs have been observed to be heritable in arthropods, but their role in the context of rapid evolution of insecticide resistance remain poorly understood. Here, we discuss evidence supporting how: firstly, insecticide-induced effects can be transgenerationally inherited; secondly, epigenetic modifications are heritable; and thirdly, epigenetic modifications are responsive to pesticide and xenobiotic stress. Therefore, pesticides may drive the evolution of resistance via epigenetic processes. Moreover, insect pests primed by pesticides may be more tolerant of other stress, further enhancing their success in adapting to agroecosystems. Resolving the role of epigenetic modifications in the rapid evolution of insect pests has the potential to lead to new approaches for integrated pest management as

well as improve our understanding of how anthropogenic stress may drive the evolution of insect pests.

1.2. Introduction

The pesticide treadmill describes how agricultural insect pests evolve resistance in response to frequently used pesticides, rendering them ineffective. Pesticides are pervasive in agriculture, and are a major selective force driving the evolution of insect pests in agroecosystems [1]. Although insecticide resistance has been documented in a wide range of insect pests [2] and the genetic basis of major gene resistance has been mapped in key pests for select insecticides [3], the broader evolutionary processes that give rise to insecticide resistance remain poorly understood [4,5]. Farmers and entomologists have observed that insecticide resistance increases with the frequency of exposure to particular insecticides [6–8], but can be lost following the relaxation of insecticide use [9– 11]. The rapid gain and loss of resistance appears to occur far more rapidly than expected based upon mutation rates [12,13], suggesting that insecticides themselves may increase the rate of mutation or cause physiological changes in pest organisms [5]. One possible explanation that has been relatively unexplored is that the evolution of insecticide resistance results from epigenetic modifications, which are heritable and influence gene expression without changing the underlying DNA sequence. The evolution of insecticide resistance has been considered an evolutionary paradox [5], in that pest species which have experienced repeated genetic bottlenecks due to invasion and selection remain able to adapt very rapidly, despite limited genetic diversity. The same insect pests have evolved resistance to insecticides in all of the major classes [14],

and are expected to evolve resistance to future chemistries [15]. Extreme genetic bottlenecks also do not appear to limit the likelihood that insecticide resistance evolves. For example, all Colorado potato beetle (Leptinotarsa decemlineata Say) populations in Europe are descended from the introduction of a single female, or single mtDNA haplotype [16]. Despite this strong historic bottleneck, L. decemlineata populations in Eurasia have evolved resistance to a wide range of insecticides in Europe, the Middle East, and East Asia [6,17,18]. There is a seeming inevitability of insecticide resistance developing in pests, where new phenotypes arise following environmental stress at rates that may not be explained by natural selection. Indeed, Skinner et al. [19] argued how epigenetic processes fit within a neo-Lamarckian framework, because environmental epigenetic patterning can influence transgenerational transmission of phenotypic variation. By influencing epi-genetic modifications, xenobiotic and environmental stressors can directly influence the phenotypic responses of organisms to their environment. Epigenetics is the field of study that examines how environmental factors influence heritable changes in gene expression. There are several epigenetic mechanisms that are heritable and could underlie transgenerational effects of insecticides: DNA methylation [20], histone modifications [21], and heritable noncoding RNA [22]. Here, we discuss evidence supporting how: firstly, insecticide- induced effects can be transgenerationally inherited; secondly, epigenetic modifications are heritable; and thirdly, epigenetic modifications are responsive to insecticide-induced stress. We draw on other model systems from a diverse body of literature, including genetics, epigenetics, and toxicology to identify gaps in our understanding around the evolution of insecticide

resistance in insect pests. We close with a discussion of the implications of epigenetic processes for insect fitness in intensively-managed agroecosystems.

1.3 Insecticide-induced hormetic effects can be heritable

Insecticides not only select for insecticide resistance and point mutations at target sites, but they can also affect physiological and life-history traits [23]. In particular, exposure to sublethal dosages of insecticides can incur stress and lead to increased phenotypic variation [24]. Stress responses can lead to hormesis, a well-known phenomenon from toxicological literature, where small dosages can stimulate biological functions whereas large dosages are detrimental or lethal [24]. Hormetic responses include activation of stress response pathways in a variety of taxa from microbes, plants, and animals. They are not related to any special class of compounds, as hormetic effects have been reported for over 240 different chemical classes [23]. Sublethal exposure to insecticides can induce hormetic effects and lead to variety of positive life history effects, such as mating success [25], fecundity [26], and body size [27]. By positively influencing traits associated with fitness, hormetic effects may play an important role in pest evolution. There is evidence that individuals exposed to stressful conditions, either abiotic or biotic, can prime gene expression in their offspring to be able to better tolerate stress [28,29]. Insecticides have been shown to induce transgenerational insecticide induced hormetic effects, but thus far the results have been difficult to interpret. For exam- ple, Myzus persicae aphids treated with sublethal levels of imidacloprid produce offspring that survive longer when exposed to food/water stress, but tolerance to insecticide stress is unchanged [30]. Similarly, although sublethal levels of precocene (an

antagonist to Juvenile hormone) stimulate reproduction in M. persicae, the results are not passed on to subsequent generations [31]. Although chemical-induced hormesis has been reported from many groups and these changes have also been reported to be inherited [23] the genetic, epigenetic, and toxicological basis of hormesis is still poorly understood [5,32].

1.4 Epigenetic modification and transgenerational inheritance

Epigenetic modifications have been shown to be heritable [20]. DNA methylation, the addition of a methyl group to the 5 carbon position of cytosine a nucleotide (usually the cytosine in CpG dinucleotides), is a well-documented mechanism of epigenetic inheritance that can influence phenotypic variation (Table 1), and is found in most, if not all, orders of insects [32]. Methylation in insects is largely found within coding regions, and is closely linked with gene expression and alternative splicing — where a single gene can generate a diversity of gene transcripts of differing length, based on which exons are translated [33]. Methylation can occur at any location in the genome, but the effects of DNA methylation vary based on its location in the genome (Figure 1): (a) changes in DNA methylation at the promoter region can influence gene expression in downstream genomic regions [34], (b) methylation suppresses gene expression of transposable elements (TEs, which are mobile genetic elements responsible for the majority of mutations in many genomes) and prevent TE mobilization [35], and (c) gene body methylation can increase gene expression [32], as well as an increase in the number of alternative splice variants [36]. Changes in methylation patterns in arthropods can be associated with changes in levels of resistance to insecticides. Myzus persicae, can gain

insecticide resistance through the duplication of esterase genes and subsequent overexpression of esterases [37]. After suspending insecticide exposure, extra copies of esterase genes can be methylated, leading to a loss of resistance. It is possible that these aphid populations could quickly become resistant again following demethylation of these amplified genes. Histone modifications include additions of acetyl or methyl groups on the histone proteins around which nuclear DNA is wrapped, which can influence gene regulation and expression [38]. The full effects of these modifications are not well known, especially in arthropods. However, it does appear that some histone modifications are able to be transmitted transgenerationally [39]. Different noncoding RNA (ncRNA) [22] can be inherited through either the male or female gametes, though most current research does not incorporate analysis of heritable RNA. Certain types of small RNA can direct and maintain DNA methylation and histone modification, and therefore affect chromatin structure [40]. DNA methylation, histone modifications, and ncRNAs form a constellation of interacting effects that result in a phenotypic response [41]. To fully understand how epigenetic modifications influence transgenerational phenotypic inheritance, it would be optimal to assess all three mechanisms simultaneously through concurrent small RNA-seq, bisulfate-treated DNA-seq, and histone modification assays, in as many tissues and individuals as possible. Ideally, multiple generations would be sequenced, to determine if changes in epigenetics and gene expression differ consistently between treatments. Because the cost of sequencing is the major limiting factor for these studies, projected lower sequencing costs in the future should enable these types of studies.

1.5 Epigenetic modifications are responsive to xenobiotic stress

Exposure to insecticides and other xenobiotic compounds can alter DNA methylation status in arthropods, and these epigenetic changes can persist for at least several generations [20,42,43]. Table 1 lists a number of examples of stress leading to epigenetic changes in arthropods. Studies focusing on insects are few in number, so our scope is broadened to include examples from aquatic ecotoxicology literature, which includes a number of non-insect arthropods. Oppold (2015) found that expo-sure of mosquitoes to a fungicide leads to heritable changes in methylation and decreases in sensitivity to imidacloprid, an insecticide. Methylated cytosines also spontaneously deaminate, becoming thymines, at a higher rate than non-methylated cytosines, which can lead to higher mutation rates in methylated regions [44]. If genes that are associated with resistance are methylated, which leads to increased expression and increased mutation rate, then genes that are most upregulated in response to insecticide resistance may also be the most likely to experience spontaneous deamination. Both the role of histone modifications and small RNA in modifying epigenetic responses to toxins are less under-stood than DNA methylation in arthropods, though it has been shown that methylation and histone modifications tend to be co-located in the genome [38]. Kishimoto et al. [45] showed that parental hormetic responses to oxidative stress can be epigenetically transmitted to descendants via histone modifications. A wide range of environmental chemicals, such as heavy metals, air pollutants, dioxins, and endocrine disrupters, can alter his-tone modifications [46], but it is unknown whether these changes are heritable. We have not found any studies on arthropods examining if insecticides can induce transgenerational small RNAs responses. Small RNAs have been found to interact with

histone modifications [47], so changes in small RNAs may be implicated in the trans-generational inheritance of stress phenotypes as well.

1.6. Implications for transgenerational effects on insect fitness in agroecosystems

We hypothesize that pesticide use can directly and indirectly drive the evolution of insect pests in agroecosystems via epigenetic processes (Figure 2). Pesticides may directly stimulate the expression of advantageous phenotypes, which may be underwritten by epigenetic modifications. Continued insecticide use on populations developing resistance would thus operate as 'natural selection' and selectively increase the frequency of insect phenotypes that are adaptive to pesticides. Indirectly, pesticide use may maintain stressful environments that hormetically prime insect pests to become more tolerant of stressful conditions. For instance, sublethal exposure to insecticides can influence adult body size of the L. decemlineata [23], which may allow insect pests to increase their tolerance to overwintering conditions [48]. Insecticides can also increase female fecundity [49] or propensity to mate [25], which can increase population size. The phenotypic traits of insect pests that allow them to thrive under insecticide exposure may also facilitate global invasions. For example, L. decemlineata is a globally-invasive pest that is expanding its range northwards into the Arctic Circle [48]. Insecticide exposure appears to stimulate the beetle to invest more in fat bodies and have a higher metabolic rate than control beetles [50]. Although the higher metabolic rate and larger fat bodies may enable beetles to better detoxify chemicals, higher fat body reserves enable small individuals to overwinter successfully [51,52]. For example, sublethal applications of the pyrethroid deltamethrin on resistant L. decemlineata populations can have stimulatory

effects rendering exposed individuals larger which is also inherited to the next generation (Lindstrom, unpublished data). To date, most of the research examining the role of pesticides or xenobiotics in epigenetic change come from the field of aquatic toxicology [53], where environmental exposure to toxins can be highly variable and difficult to predict. By contrast, pesticide use in agroecosystems is intentionally part of an active pest management system, where insect responses to stresses can cause positive feedbacks on subsequent management decisions. Agroecosystems are also highly controlled systems, which allows for greater experimental control for field and landscape level studies. Along these lines, it would be important to know how epigenetic responses to the same insecticides may vary among individuals, populations, and species. Such information would help provide insight on whether epigenetic responses can be broadly predictable across individuals and species, and possibly, how pesticide resistance may be better managed. A combination of new genomic tools, epigenetic assays, and computationally-intensive approaches may allow us to better understand to what extent epigenetic responses within insects help drive the pesticide treadmill.

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1.9 Figures

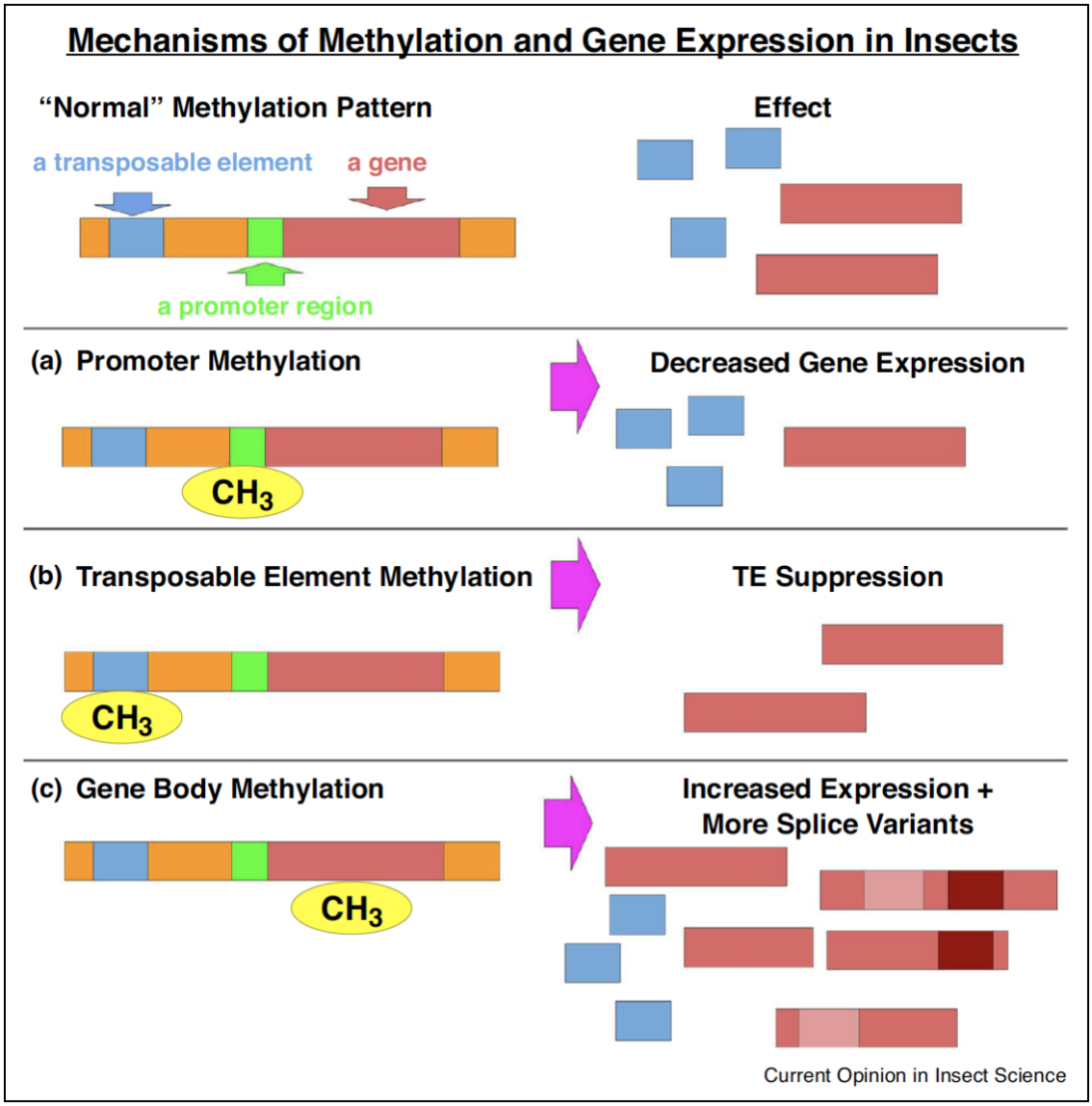

Figure 1.1: Examples of how changes in methylation status of in different gene regions can effect gene expression. Compared to the 'normal' unmethylated region, (a) has promoter methylation, leading to decreased gene expression; (b) exhibits methylation in transposable element regions, leading to those elements not being expressed, and (c) shows gene body methylation as found in arthropods, leading to increased gene expression as well as an increased variety of splice variants in those transcripts.

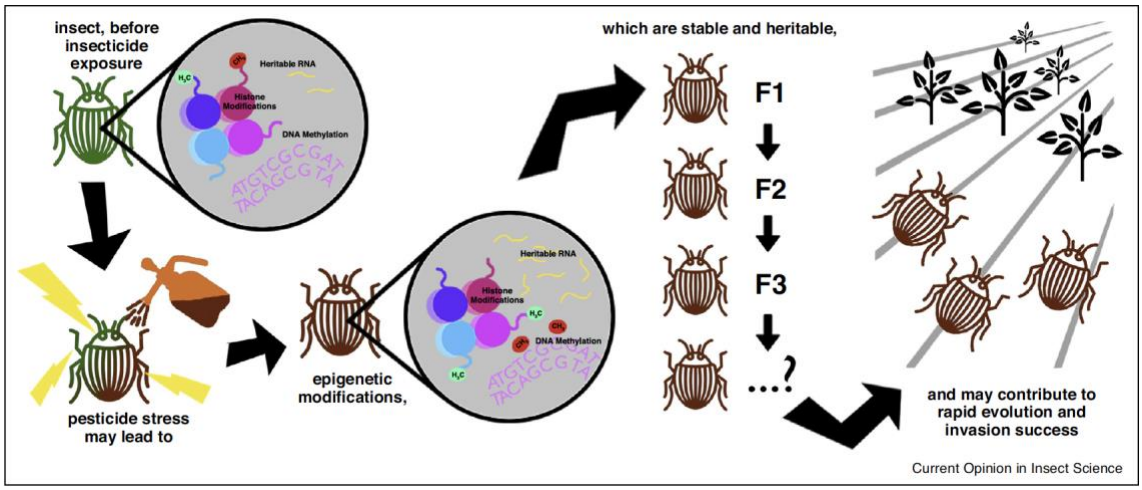

Figure 1.2: How exposure to a stressor may lead to heritable epigenetic changes that could lead to stress-resistant phenotype in an invasive agricultural insect pests.

1.10 Tables

Table 1.1: Examples of epigenetic alterations following exposure to anthropogenic and 'natural', (non-anthropogenic) stress.

Table 1

Examples of epigenetic alterations following exposure to anthropogenic and 'natural', (non-anthropogenic) stress.

	Species	Treatment	Phenotypic effects	DNA methylation	Histone modifications	Transgenerational effect	Reference
Anthropogenic stressors	*Daphnia magna*	Vinclozolin (fungicide)	Body size	Reduced methylation	n/a	N	[20]
	Daphnia magna	5-Azacytidine (demethylating agents)	Reproduction, body size	Reduced methylation	n/a	Y	[20]
	Myzus persicae (Green Peach Aphid)	Imidacloprid (insecticide)	Changes in gene expression, including heat shock protein	Increased, decreased, or no change based on concentration	n/a	Y	[31•]
	Aedes albopictus (Asian Tiger Mosquito)	Genistein (phytohormone)	Decrease in sensitivity to imidacloprid	Cautious decrease	n/a	Y	[54]
	Aedes albopictus (Asian Tiger Mosquito)	Vinclozolin (fungicide)	Decrease in sensitivity to imidacloprid	Cautious increase	n/a	Y	[54]
'Natural' stressors	*Artemia* sp. (brine shrimp)	Heat stress	Increased Hsp70 production, heat tolerance, and resistance versus pathogens	Changes in methylation	Histones H3 and H4 acetylation	Y	[55]
	Daphnia magna	Zinc	Changes in gene expression	Reduced methylation	n/a	Y	[56••]
	Daphnia magna	Toxic cyanobacterium *Microcystis aeruginosa*	n/a	Differential methylation primarily in exonic regions, enriched for serine/threonine amino acid codons and genes related to protein synthesis, transport and degradation, in genes susceptible to alternative splicing in response to Microcystis stress	n/a	N	[57]
	Plutella xylostella (Diamondback Moth)	Endoparasitoid	Altered gene expression	Reduced methylation	n/a	N	[58]
	Drosophila melanogaster (Fruit Fly)	Heat shock or osmotic stress	n/a	n/a	Heterochromatin disruption	Y	[58]

CHAPTER 2: PESTICIDE DURABILITY AND THE EVOLUTION OF RESISTANCE: A NOVEL APPLICATION OF SURVIVAL ANALYSIS

2.1 Abstract

Arthropod pests are widely perceived to evolve resistance to insecticides at different rates. Although widespread "successful" species are assumed to evolve quickly and minor pests slowly, few studies have utilized published data on resistance events to test for differences among species. Using 532 records from the Arthropod Pesticide Resistance Database covering 20 species, we applied a survival analysis to model the number of generations from insecticide introduction to the first report of arthropod resistance, providing one of the most comprehensive analyses of this question to date. Our approach tested: 1) whether successful pests evolve resistance faster than close relatives, 2) whether species differ significantly in the time to demonstrate resistance, and 3) whether different insecticide classes differ in durability (length of time an insecticide is used before resistance arises). We found that species differed significantly in the amount of time it took for resistance to be reported. Overall, the median duration between the introduction of an insecticide and the first report of resistance was 66 generations (95% c.i. 60-78 generations), and highly-resistant arthropods did not evolve resistance faster than their relatives. Insecticide durability did not differ by the mode of action or year of introduction. Arthropod species significantly varied in how rapidly they evolve resistance to new insecticides, regardless of their chemistry. Visualization of the history

of insecticide resistance provides information to be used for understanding how pesticide resistance evolved and how it can best be managed.

2.2 Introduction

The ability of insects to evolve resistance to insecticides is one of the most significant factors contributing to the cost of managing arthropod pests[1,2], with current estimates of global pesticide expenditures at $40 billion per year[3]. Pesticide or field-evolved resistance is a genetically based decrease in susceptibility of a population to a pesticide caused by exposure to the pesticide in the field[4].While case studies of insecticide resistance have proven fruitful in understanding individual events of insecticide resistance, the broader evolutionary processes that give rise to insecticide resistance remain poorly understood[5,6]. For instance, species are thought to evolve insecticide resistance at different rates, but the conventional wisdom is only based on anecdotal observations rather than a statistical approach across aggregated data. Aggregating data across insecticides and species may provide an avenue to understand the trends that lead to resistance, and could contribute to novel approaches to slow the rate of evolution of resistance. A broad look at the history of the evolution of insecticide resistance in a variety of species may provide valuable insight into wider trends of development of resistance within and between species. The majority of research on insecticide resistance has tended to focus on either elucidating a biochemical or genetic mechanism of resistance to a specific insecticide class[7,8] or modeling the spread and growth of resistance in specific field and species contexts[9]. While these approaches are crucial for understanding how insecticide resistance can evolve and spread, they do not

provide insight on the pace of evolution for insecticide resistance. By applying a survival analysis approach to aggregated data, we tested for general patterns in the development of insecticide resistance, as well as testing for differences in durability of various insecticide MoA (modes of action). Our approach reveals that there are broader evolutionary patterns in the pace of insecticide resistance across individual species, and yields generalizable insights for pests where resources for detailed molecular or genetic studies are limited. While a number of genetic, ecological and operational factors intervene in the development of resistance10, the rate of evolution of insecticide resistance may vary considerably between species11,12, but the literature lacks rigorous comparisons between species across geography and pesticide chemistries. Differences in 'evolvability' has been proposed as a possible mechanism explaining variation in the rate of evolution among species, where certain species are able to more rapidly evolve13. The underlying mechanisms responsible for variation in evolvability in response to insecticides differences remain unknown. Evolvability itself could be influenced by inherent genetic differences, such as different mutation rates between species14, biochemical differences stemming from dietary differences15, and differences in initial gene frequency. Also, the number of generations per year and population size could also influence the likelihood that populations evolve resistance; species with large populations have more chances for mutations leading to resistance16. Admittedly, comparisons of the rate of insecticide evolution among agricultural pests is challenged by the fact pest species may be exposed to different numbers, chemistries, and rates of insecticides, in completely different environmental contexts. For example, a widespread pest may be frequently exposed to a rotation of several insecticides across multiple continents17, while a pest with a more

restricted distribution may only be sporadically exposed to a single chemical18. Therefore, attempts to compare species with such varied insecticide exposure histories is challenging due to lack of comparable data. We developed a novel approach for analysis and visualization of the history of emergence of insecticide resistance in insect pests. We adapted a survival analysis methodology to assess durability, the length of time before resistance to the chemical is reported in a species. Therefore, we essentially asked how long an insecticide "survived" against a pest species. We used survival analysis to compare the rate of evolution of resistance among species, and to test patterns underlying the evolution of insecticide resistance in arthropod pest species, using the Arthropod Pesticide Resistance Database (APRD), which contains a large global database of the reported incidents of insecticide resistance. We tested if insecticides differed in their durability against the most highly resistant pests (pests that have most frequently developed resistance) compared to other arthropods. To account for phylogenetic relatedness and underlying biological differences among arthropod pests, we compared the ten species with the highest number of resistances with ten of their closest relatives found within the database. We used the APRD database to ask the following questions: 1) Does insecticide durability differ between the selection of the most resistant arthropods vs. their closest relatives? 2) How variable are pest species in their rate of resistance evolution? 3) Do insecticidal chemistries, which vary by mode of action, differ in terms of their length of effectiveness? 4) Do insecticides released more recently have shorter lifespans, possibly due to cross-resistance? Understanding the history of insecticide resistance by integrating data from aggregated databases could prove a valuable tool in the ongoing task of managing insecticide resistance in arthropod pests and will provide

insight on common evolutionary patterns that drive the pace of evolution of insecticide resistance.

2.3 Materials and Methods

2.3.1 Data Sources

We gathered data fromtwo sources: the Arthropod Pesticide Resistance Database (APRD)19 and the Pesticide Properties Database (PPDB)20. In order to test if insecticide durability differs between highly resistant species and their relatives, we selected ten arthropods that were resistant to the highest number of insecticides as reported by the IRAC database21, and then found their closest relative that had a record of resistance in the APRD. We use the term insecticide here to apply to both insecticides and chemicals targeting other arthropods, such as acaricides. For each arthropod listed in Table 1, we retrieved all citations documenting the resistance of that species to each insecticide. We selected the earliest report of resistance for each insecticide/arthropod combination, which was considered the initial resistance event. To determine when an insecticide was first used, we manually retrieved introduction dates for each insecticide from the PPDB. To calculate the time it took a species to evolve resistance to an insecticide,we subtracted the introduction date from the first report of resistance. Because arthropod species vary in the number of generations per year, each species was normalized based on its average number of generations per year (Supplementary Table 1). Overall, we analyzed a total of 532 resistance cases distributed among the 20 species of interest. We chose the ten resistant species were chosen because they have been exposed consistently to many insecticides. We note that some important global pests, such as the mosquitoes Aedes

aegypti (L.) and Culex quinquefasciatus (Say)22, were not included in this analysis.While these species have high numbers of cases of resistance, (a case of resistance is defined as the field-evolved resistance of one species to an active ingredient in a geographical area in a given23, they have not developed resistant to as many insecticides (active ingredients) as the selected species. However, the introduction date of an insecticide does not guarantee immediate use against a specific pest and detailed records were not readily available. Therefore,we assumed thatwidespread pestswere more likely to be exposed to novel insecticides closer to their 'release date'. In our analysis, we likely overestimate the time until the evolution of resistance for two reasons. First, the data is based on the publication date of a report, which includes the duration of time between discovery of resistance and publication of a report of that resistance. Second, the introduction date of an insecticide does not necessarily indicate that the insecticide was used against that pest in the first year. Finally, our analysis is limited in that it does not include data for resistance events for recently-released insecticides because resistance has not yet occurred or been reported, even though arthropods may be in the process of evolving resistance.

2.3.2 Analysis

We used survival analysis to estimate the durability of insecticides against individual pests. Survival analysis allows the prediction of the likelihood that an event will occur over time. It is commonly used in public health to estimate how long a patient will survive facing a certain disease, or how long a mechanical component will last until failure24. Survival analysis is well suited to analyzing the durability (time an insecticide

can “survive” against a certain pest before resistance emerges in the population) of insecticides. We fitted models in R (v 3.3.3,25) using the survival package26(v. 2.41-3) and the survminer package27(v. 0.4.0). We used Kaplan–Meier estimators to fit the following parameters separately, including species status (highly resistant vs not-highly resistant), mode of action, and year of introduction. For testing the statistical significance among groups, we used log-rank tests, an established non-parametric method for comparing survival distributions28. To calculate the useful lifespan of insecticides versus specific insect pests, we determined the Kaplan–Meier estimates of survival curves and compared them using a log-rank test. First, an overall survival function was fit using all 532 resistances cases to yield an overall estimate of the “global” survival of insecticides when used against arthropods. To test whether the rates of resistance differed between the most resistant species and their relatives, we compared the survival curves of the insecticides for the two groups. We also compared the survival curves for the number of generations until resistance developed in each of the twenty species, with a separate curve for each. We then tested if the durability of each mode of action (MoA) differed from one another. Finally, we examined the role of cross-resistance in contributing to insecticide durability by examining the year of introduction of insecticides, to determine if insecticides released more recently had shorter lifespans before the evolution of resistance.

2.4 Results

Overall, we found that insecticide durability significantly varied against our twenty selected species (Figure 1, $p < 0.0001$). The survival curves in Figure 1 show the

percent of insecticides that are remain without a report of resistance at each time point. Table 3 shows the mean number of generations until resistance develops in the included species. Diabrotica virgifera, Blatella germanica, and Leptinotarsa decemlineata appear to evolve the fastest. The most resistant species did not differ from their relatives in terms of the speed at which resistance is reported (Figure 2, p =NS). The counts vary between the two groups because the most resistant species have evolved resistance to more insecticides. For example, in Figure 2, about 37% of insecticides have no reported resistance against them at 100 generations. In other words, at 100 generations: 132 insecticide/species pairings have no reports of resistance, out of 419 total for the most resistant species, and 51 out of 113 for their relatives. The variation present between species is rather remarkable, with some species, like the German cockroach (Blatella germanica L.) and the Western corn rootworm (Diabrotica virgifera LeConte), developing resistance to all included insecticides in under 100 generations, while others, like the housefly (Musca domestica L.) taking up to 800 generations to develop resistance. We found that pesticide durability did not significantly vary amongMoA (Figure 3; p =0.071). Although we did not find a clear statistical significance, the result is nearly significant, possibly due to limited data, and increased numbers of records may alter the results. Some insecticides, such as endosulfan and methoxyclor (organochlorines), lasted upwards of 700-800 generations against some species, while others were rather short lived, such as thiacloprid (a neonicotinoid) and etofenprox (a pyrethroid). Table 2 shows the average number of years until resistance was reported for all insecticides present in this study for which multiple reports of resistance were present. Overall, the median duration between the introduction of an insecticide and the first

report of resistance was 66 generations (95% c.i. 60-78 generations), or about 14 years (Figure 4). Comparing the median time to resistance for pesticides introduced in each year by the year of introduction (Figure 5, with each dot representing all pesticides introduced in that year), there is not a significant relationship between them (p=NS), indicating that has not been a change in the length of pesticide survival (or how long they last) over time. Cross resistance does not appear to be a significant driver of resistance trends. In Figure 6(a-t), we present data showing a graphical timeline of evolution of insecticide resistance for each included pest species, showing both the history of introduction of insecticides and the times at which resistance emerges. These figures provide a way to quickly gain insight on the evolution of pest resistance in each particular pest. For example, in the graph representing the Colorado potato beetle (6d) (Leptinotarsa decemlineata Say), the early introduction of hydrogen cyanide and rotenone is visible in the upper left, (it took over 100 generations for resistance to these compounds to be reported) and in the lower right, the recent cluster of neonicotinoids introduced in the 1990s and early 2000s, with resistance to these compounds reported in under 20 generations. As another example, DDT was introduced as an agricultural insecticide in 1945, but the number of generations until resistance was reported varies considerably between species – from 22 generations (1955) for L. decemlineata to 375 generations (1969) for Myzus persicae. An interactive version of these data is available here: http://www.kristianbrevik.com/illustrating-thepesticide- treadmill.

2.5 Discussion and Conclusion

Although we did not find evidence that the most resistant species evolved resistance faster than their counterparts when aggregated into two groups, we did find evidence that individual species differ in the time they take to evolve resistance to insecticides (Figure 1). These results provide benchmarks for the expected durability of various insecticides. There are several possible explanations for why insect species appear to evolve at different rates. First, species could vary genetically, in terms of initial gene frequency, mutation rate29,30, epigenetic responses31, or transposable element activity32–34. Insect pests may also vary in terms of the degree of exposure to insecticides. For example, based upon their life history characteristics and food preference, household pests may be exposed to very high doses via poisoned traps, while crop pests may be exposed through foliar sprays35–37. Species that tend to outbreak frequently would be expected to incur higher insecticide dosages and applications that are more frequent. Species are exposed to different numbers and quantities of insecticides, and the reporting of insecticide resistance likely differs between species, based on commercial or medical importance of a pest, or based upon the personal interests of entomologists. Unfortunately, these underlying factors are not reflected in the APRD database. Our data suggest that there are differences among species in evolvability. For example, the housefly (Musca domestica) appears to develop resistance to insecticides much more slowly than other species (Figure 1). However, considering the possibilities outlined above, the housefly may evolve resistance more slowly because: 1) the species is inherently less evolvable due to a biological difference, 2) the housefly may encounter insecticides in houses and in and around livestock enclosures, enclosed facilities which

may intensify selection pressure and contribute to a lack of gene flow38, 3) the fly is perhaps of less economic importance than agricultural pests39, and so fewer insecticides are applied against it, or 4) fewer resources are spent detecting resistance. Researchers familiar with each individual species will be able to contribute insight into the factors contributing to the survival curve analysis of each species. By using these factors as covariates in a statistical analysis, a much larger dataset could detect the role of these factors in contributing to insecticide resistance and the durability of certain insecticides. We acknowledge that databases reliant on self-reporting can be inherently flawed, due to the differences in funding and number of researchers focusing on different pest species, however, the methods proposed here provide valuable insight into the evolution of pesticide resistance and may be of use when applied to other datasets. We were not able to find introduction dates for some of the reported insecticides within the PPDB, particularly the Bacillus thuringiensis subtypes. However, Bt genes incorporated in crops has reduced the number of pesticide sprays in Diabrotica virgifera, Bucculatrix thurberiella, Helicoverpa zea, and data for these pests has likely be affected by the introduction of these genetically engineered crops. Because we have more data points for the species with more resistance, estimates of rates of resistance evolution could be biased. As an extreme example, there are three members of the order Blattodea (cockroaches) in the APRD, and the German cockroach (Blatella germanica L.) has 37 recorded resistances to insecticides with available release dates, while the Oriental cockroach (Blatta orientalis L.) and the brown cockroach (Periplaneta brunnea Burmiester) each have only one. Nevertheless, by combining many species together, these data gaps and biases should have less impact on our results40. As far as we know,

this is the first study to use a survival analysis approach to investigate global trends in the evolution of insecticide resistance and insecticide durability. Further studies focused on experimental evolution and resistance to a variety of insecticides within one species may provide more insight on the rate of evolution of insecticide resistance, including data on which resistance events are practical for field management. To truly answer the question of different rates of evolution, much more data is necessary, especially fine-grained species-specific data including information on development time. Analysis focused on examining when a specific species developed resistance to insecticides, including multiple resistance events separated geographically, would enable the analysis of repeated evolution of resistance to the same compound in separate populations of the same species. Additionally, data on actual levels of field exposure for specific pests would provide insight on how particular factors are related to the evolution of resistance for specific populations. In conclusion, we present evidence that arthropod species do indeed evolve insecticide resistance at different rates, although the specific mechanisms by which this happens remain unclear. The median time from introduction until resistance occurred was 66 (95% c.i. 60-78) generations, about 14 years. While the survival curve of durability shown in Figure 4 is not generated using all insecticides against all arthropods, it is likely to be a close approximation of the durability curve of insecticides in aggregate. This curve may be of interest to those studying the development of novel insecticides, as it may provide a rough model on which to base expectations of insecticide longevity in the field. The fastest cases of resistance happened within a year. For example, Blatella germanica evolved resistance to etofenprox in 1988, a year after the chemical was introduced. Similarly, the European red mite, Panonychus ulmi

developed resistance to parathion 1951, and the Two-spotted spider mite, Tetranychus urticae developed resistance in 1974 to permethrin, both after the insecticide was used for a single year.). Further survival analyses using either published reports or experimental evolution in different pest species may yield results valuable for the management of individual species. Survival analysis methods provide a novel way to understand the range and scope of resistance evolution across pests. As more data becomes available and improves, these methods may provide a valuable path towards perceiving underlying patterns. We hope that this analysis and accompanying figures will prove valuable in understanding the history and tempo of the evolution and reporting of insecticide resistance and the durability of different classes of insecticide.

2.6 Acknowledgements

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2.7 Supporting Information

Supporting information may be found in the online version of this article.

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2.9 Figures

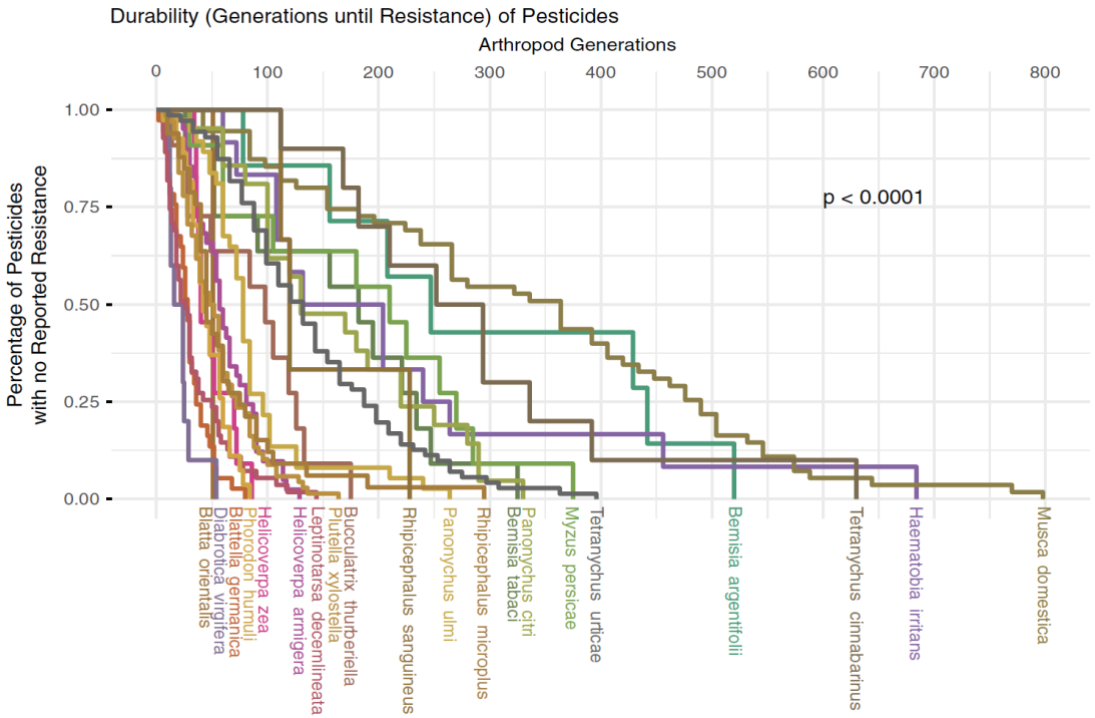

Figure 2.1: Individual survival curves (confidence intervals omitted for visual clarity) of all twenty included species.

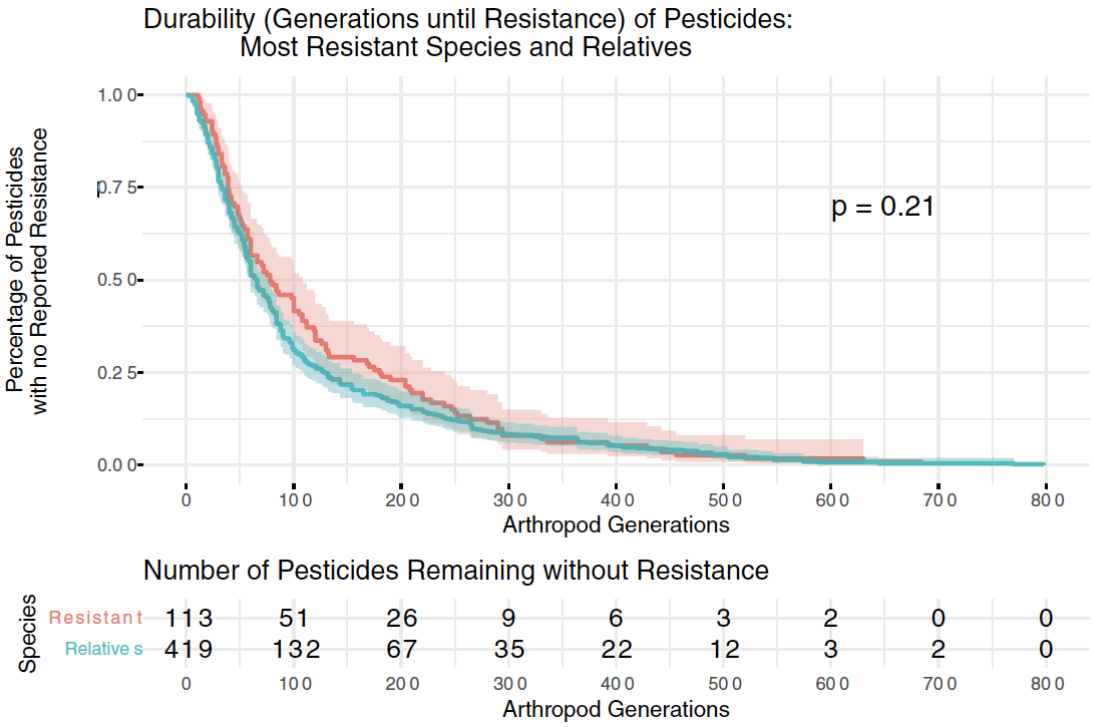

Figure 2.2: Comparison of survival curves of pesticides against the most resistant arthropods and their relatives. There is no significant difference between them.

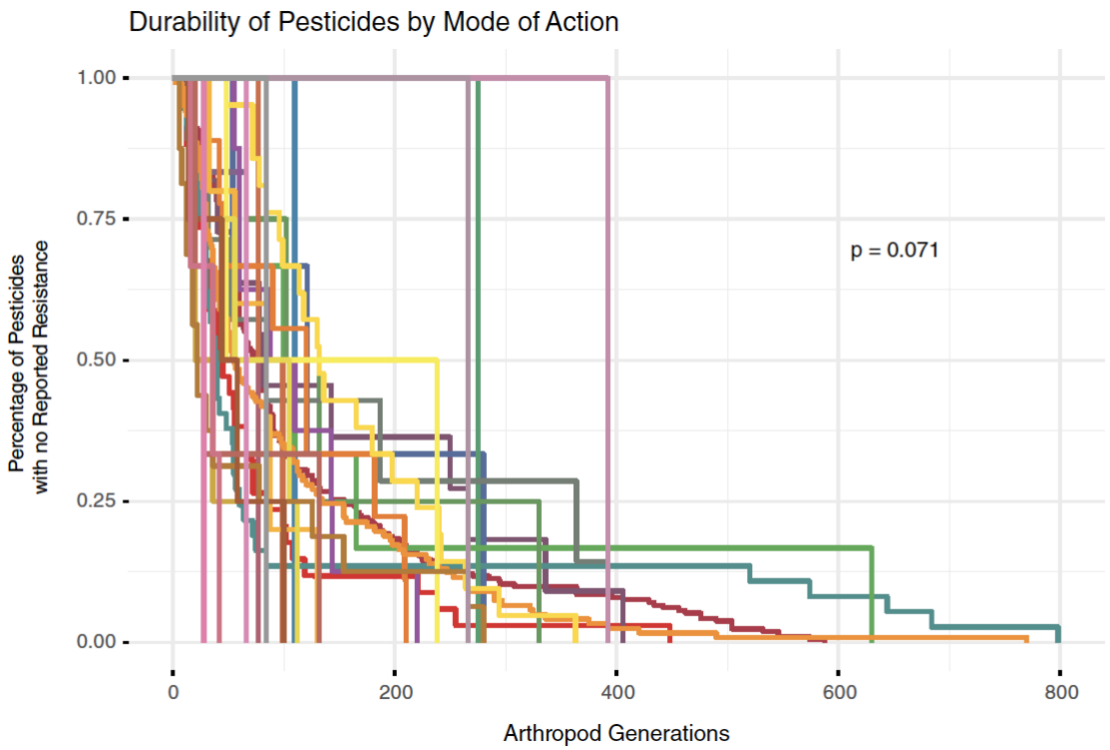

Figure 2.3: Survival curves showing lifetimes of pesticides by Mode of Action (MoA).

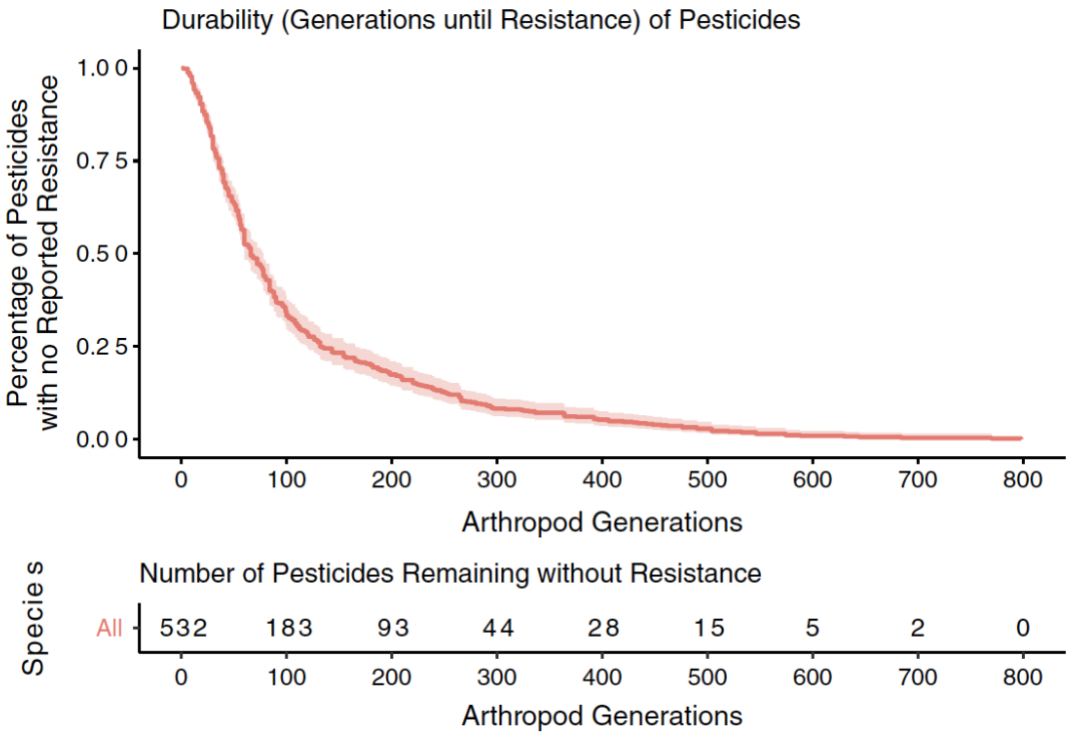

Figure 2.4: The overall survival curve for all pesticides against these twenty species, with a median of 66 (95% c.i. 60-78 generations) (equivalent to about 14 years) from introduction of a pesticide until resistance is reported. This figure (and following figures) can be read as: Percent of pesticides remaining without reported resistance (y-axis) after (x-axis)-number of arthropod generations have occurred.

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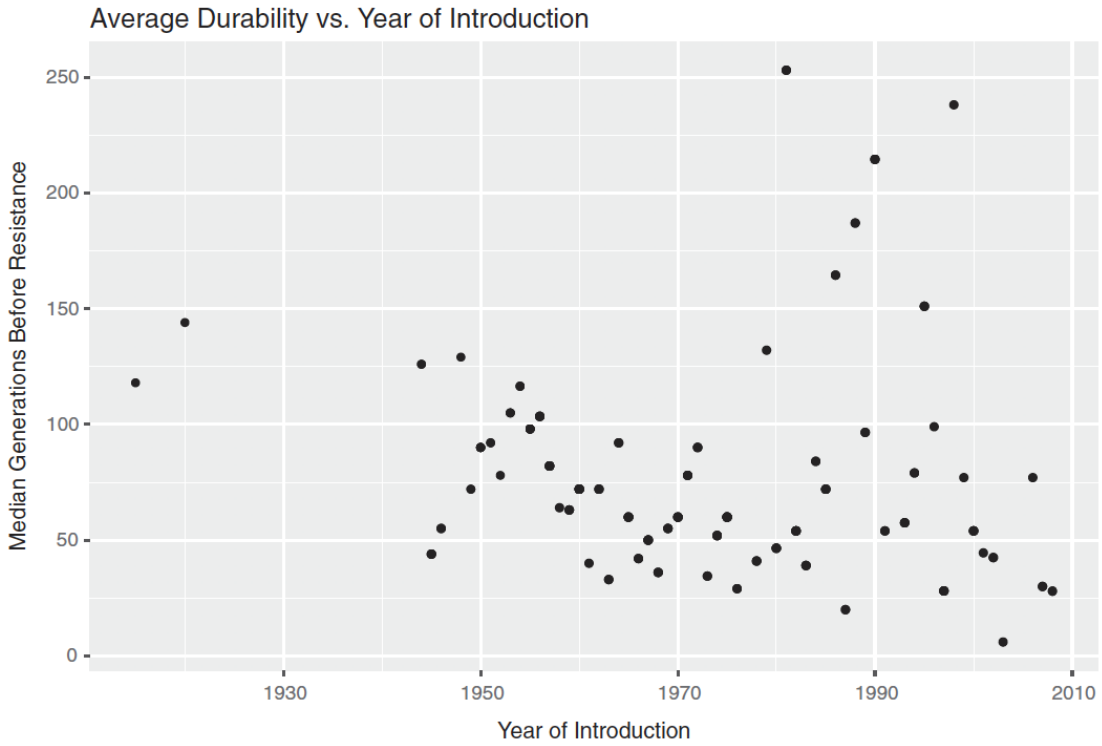

Figure 2.5: Median length of time until resistance was reported by the introduction year of a pesticide.

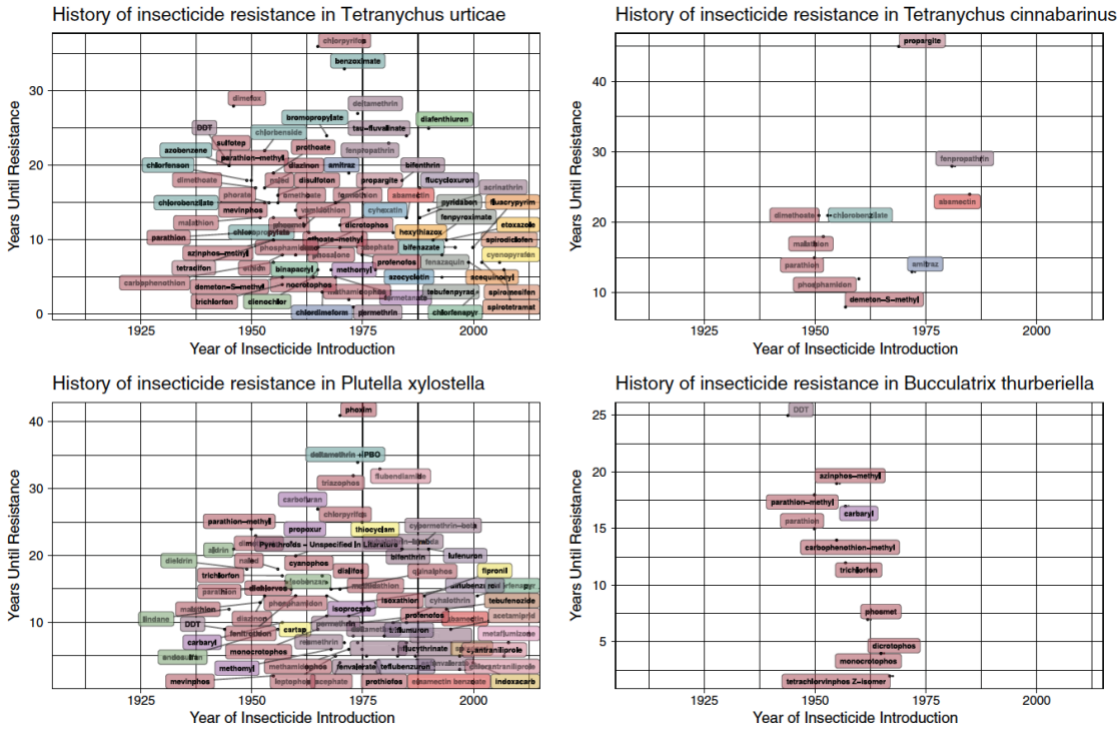

Figure 2.6: Plots of the number of generations following introduction of a pesticide that resistance was reported for the most resistant species. Each pesticide is labelled by name, and color-coded by Mode of Action. For example, hydrogen cyanide was introduced against the Colorado Potato Beetle around 1910, and it look about 120 generations for the beetle to evolve resistance. These plots show the number of generations elapsed before resistance evolves to a specific pesticide, with the year of introduction on the x-axis, and with each Mode of Action (MoA)41 grouped by color. An interactive version of this figure, including the less resistant species, is located here: http://www.kristianbrevik.com/beetle-town-illustrating-the-pesticide-treadmill.

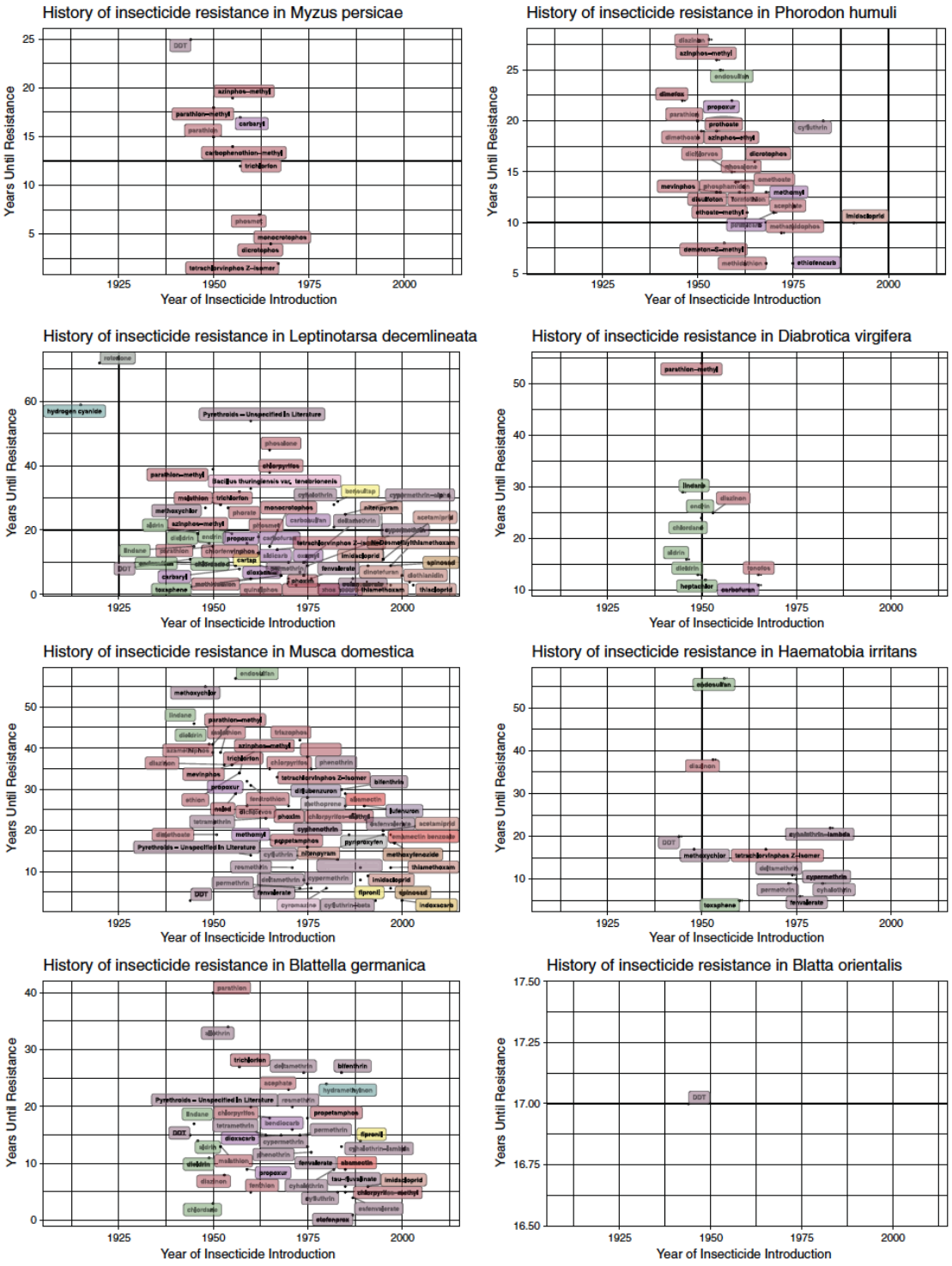

Figure 2.6: Continued

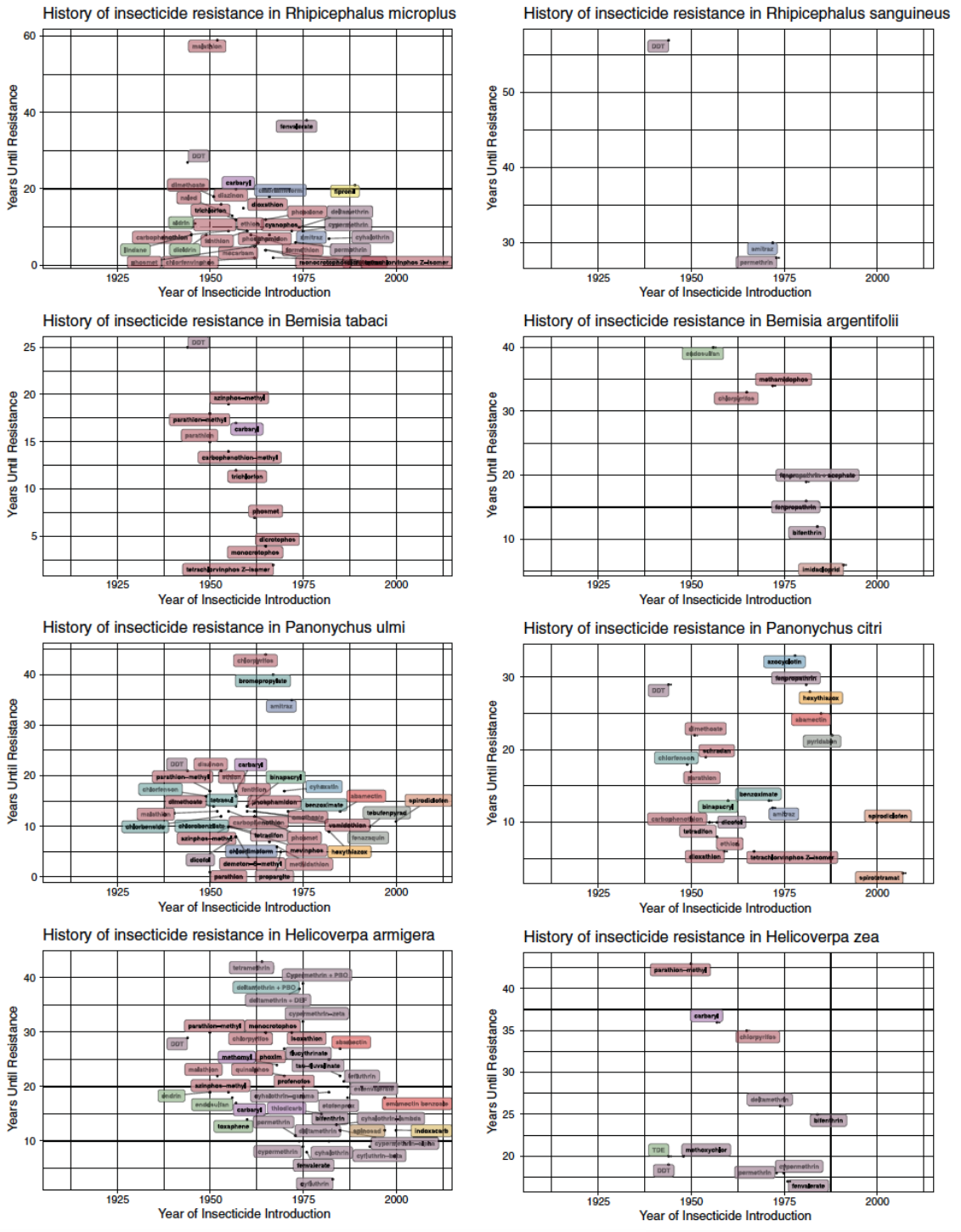

Figure 2.6: Continued

2.10 Tables

Table 2.1: Table 1 lists the species used in the analysis. The column labelled "Highly Resistant Species" refers to the ten arthropods that have evolved resistance to high number of insecticides . The column "Relative" refers to the closest relative to the highly resistant species that is also found in the APRD database. The column named, "Level of Relationship" lists the closest shared taxonomic grouping between the "highly resistant species" and it's relative

Highly Resistant Species *Latin Name* Common Name (pest of …)	Relative *Latin Name* Common Name (pest of …)	Level of Relationship
Tetranychus urticae Two-Spotted Spider Mite (vegetables)	*Tetranychus cinnabarinus* Carmine Spider Mite (vegetables)	Genus (Tetranychus)
Plutella xylostella Diamondback Moth (Brassica crops)	*Bucculatrix thurberiella* Cotton Leaf Perferator (wild cottons)	Superfamily (Yponomeutidae)
Myzus persicae Peach-Potato Aphid (peach trees, potatoes)	*Phorodon humuli* Hop Aphid (peach trees, hops)	Family (Aphididae)
Leptinotarsa decemlineata Colorado Potato Beetle (potatoes)	*D*iabrotica virgifera Western Corn Rootworm (corn)	Family (Chrysomelidae)
Musca domestica Housefly (household/livestock pest)	*H*aematobia irritans Horn fly (livestock)	Family (Muscidae)
Blattella germanica German Cockroach (household pest)	*B*latta orientalis Oriental Cockroach (household pest)	Order (Blattodea)
Rhipicephalus microplus Asian Blue Tick (livestock)	*Rhipicephalus sanguineus* Brown Dog Tick (livestock)	Genus (Rhipicephalus)
Bemisia tabaci Sweetpotato Whitefly (tomatoes, other vegetables)	*Bemisia argentifolii* Silverleaf Whitefly (pointsettias)	Genus (Bemisia)
Panonychus ulmi European Red Mite (fruit trees)	*Panonychus citri* Citrus Red Mite (citrus trees)	Genus (Panonychus)
Helicoverpa armigera Cotton Bollworm (cotton)	*H*elicoverpa zea Corn Earworm (corn)	Genus (Helicoverpa)

Table 2.2: Mean durability of insecticides included in this analysis, for insecticides where more than one report was present

Table 2. Mean durability of insecticides included in this analysis, for insecticides where more than one report was present

Insecticide Name	Mean Years Until Resistance	SD
spirotetramat	4.50	2.12
cyfluthrin-beta	6.00	4.24
indoxacarb	6.33	4.93
dicrotophos	6.83	4.92
thiamethoxam	7.00	5.66
demeton-S-methyl	7.25	1.50
chlorfenvinphos	7.50	7.78
tebufenpyrad	7.50	3.54
imidacloprid	8.00	1.87
spinosad	8.00	3.16
toxaphene	8.00	5.20
methidathion	8.25	4.50
chlorfenapyr	8.50	7.78
cartap	9.00	2.83
dicofol	9.00	1.41
fenazaquin	9.00	1.41
chlordimeform	9.33	7.77
fenthion	9.33	4.51
etofenprox	9.50	12.02
monocrotophos	9.50	9.13
phosmet	9.71	4.54
carbophenothion	9.75	2.50
tetrachlorvinphos Z-isomer	9.75	11.12
spirodiclofen	10.00	1.00
tetradifon	10.00	0.00
cypermethrin	10.13	3.80
cyfluthrin	10.25	7.80
dioxacarb	10.50	6.36
dioxathion	10.50	6.36
methamidophos	10.80	13.26
permethrin	10.90	7.69
ethoate-methyl	11.00	0.00
formothion	11.33	2.89
esfenvalerate	11.60	9.61
binapacryl	11.67	3.21
acephate	11.75	8.06
phosphamidon	11.83	2.71
fenvalerate	12.13	11.26
cyhalothrin	12.17	7.83
cyanophos	12.50	6.36
vamidothion	12.50	0.71
resmethrin	12.67	6.66
methomyl	13.20	7.66
acetamiprid	13.33	5.86
emamectin benzoate	13.33	8.96
omethoate	13.67	0.58
quinalphos	13.67	9.07
carbophenothion-methyl	14.00	0.00
chlordane	14.00	10.54
fipronil	14.25	5.32
cyhexatin	14.50	3.54
disulfoton	14.50	2.12
tau-fluvalinate	14.75	9.57
mevinphos	14.80	12.68
deltamethrin	15.00	8.59

Table 2. Continued

Insecticide Name	Mean Years Until Resistance	SD
nitenpyram	15.00	5.66
chlorpyrifos-methyl	15.50	14.85
flucythrinate	15.50	13.44
ethion	15.60	10.81
chlorobenzilate	16.00	4.58
hexythiazox	16.00	10.44
aldrin	16.20	3.96
parathion	16.27	9.21
cypermethrin-beta	16.50	7.78
chlorbenside	17.00	7.07
propetamphos	17.00	1.41
cyhalothrin-lambda	17.25	4.92
chlorfenson	17.33	1.15
pyridaben	17.50	6.36
carbaryl	17.67	7.87
carbofuran	17.67	8.33
dieldrin	17.83	11.89
fenitrothion	18.00	11.53
trichlorfon	18.00	10.45
cypermethrin-alpha	18.50	9.19
dichlorvos	18.67	6.35
abamectin	18.75	8.26
naled	18.75	7.14
profenofos	19.00	13.71
prothoate	19.00	0.00
dimethoate	19.13	2.90
endrin	19.33	4.51
azocyclotin	19.50	19.09
amitraz	19.67	10.58
azinphos-methyl	19.67	7.87
benzoximate	19.67	11.55
propoxur	20.20	8.50
diflubenzuron	20.50	10.61
isoxathion	20.50	13.44
phosalone	20.50	16.58
bifenthrin	20.71	6.78
phorate	21.00	8.49
lindane	21.17	14.08
propargite	21.67	20.82
DDT	22.06	11.87
diazinon	22.56	10.10
lufenuron	23.50	3.54
phenothrin	23.50	16.26
fenpropathrin	24.00	5.94
malathion	24.22	15.77
phoxim	24.75	14.84
dimefox	25.00	4.24
Pyrethroids - Unspecified	27.00	18.22
tetramethrin	27.00	14.42
parathion-methyl	29.09	13.11
methoxychlor	29.75	17.35
endosulfan	30.71	20.84
bromopropylate	32.00	11.31
chlorpyrifos	32.89	7.36
deltamethrin + PBO	36.00	2.83
triazophos	37.00	7.07

Table 2.3: Mean time between introduction of an insecticide and the first reported resistance case in each included species

Table 3. Mean time between introduction of an insecticide and the first reported resistance case in each included species

Arthropod	Mean Generations Until Resistance	SD
Diabrotica virgifera	22.10	12.95
Blattella germanica	29.24	17.36
Leptinotarsa decemlineata	34.40	29.89
Phorodon humuli	46.56	17.83
Helicoverpa zea	50.36	17.82
Blatta orientalis	51.00	*NA*
Plutella xylostella	54.71	33.45
Helicoverpa armigera	62.05	28.57
Rhipicephalus microplus	64.24	55.38
Panonychus ulmi	86.43	52.07
Bucculatrix thurberiella	87.18	51.57
Tetranychus urticae	141.76	80.14
Rhipicephalus sanguineus	153.33	64.79
Bemisia tabaci	161.91	95.77
Panonychus citri	163.33	87.94
Myzus persicae	186.82	110.51
Haematobia irritans	221.00	181.95
Tetranychus cinnabarinus	287.00	146.87
Bemisia argentifolii	297.14	166.55
Musca domestica	337.27	190.14

CHAPTER 3: TRANSPOSABLE ELEMENTS DIFFER BETWEEN GEOGRAPHIC POPULATIONS OF THE COLORADO POTATO BEETLE

3.1 Abstract

Insect herbivores who feed on agricultural crops show a remarkable ability to adapt rapidly to modern agroecosystems. Given that some of the most remarkable cases of rapid evolution involve insect herbivores, they are ideal for deeper inquiry into the mechanisms of rapid evolution. One mechanism of rapid evolution that has been relatively unexplored in explaining the success of agricultural insects is that of transposable elements. Transposable elements (TEs) are ubiquitous mobile DNA elements within eukaryotic genomes that play major roles in both genome architecture and the generation of genetic variation. We examined how TE content may vary among geographic populations of the Colorado potato beetle, *Leptinotarsa decemlineata*, which vary in their association with agriculture. *Leptinotarsa decemlineata* is considered one of the most widely adaptable insect species, as shown by its wide host range, broad geographic distribution, and rapid adaptation to insecticides. However, beetle populations vary in their adaptability to insecticides, with Eastern US beetle populations being more highly adaptable than Northwestern US populations. We tested if total TE content and assemblages of transposable elements differed between geographic populations of *L. decemlineata*, and if TE content differed between beetles based on geography, host plant, and neonicotinoid insecticide resistance.

Using a presence/absence matrix of transposable element locations within the genomes of 88 North American potato beetles, we found that transposable element insertion locations differ between geographic beetle populations, reflecting the biogeographic divergence of populations in this species. Among populations of North American *L. decemlineata*, beetles collected in Mexico host more unique transposable elements than beetles collected in the United States, beetles collected on buffalobur (*Solanum rostratum)* host more unique transposable elements than beetles collected on potato (*Solanum tuberosum)* and beetles in the Northwestern United States host more transposable elements than those in the rest of the United States.

Total transposable element content between *L. decemlineata* individuals differed among populations, with TE content varying among geographic populations. Transposable element content does not appear to relate to insecticide resistance.

3.2 Introduction

BACKGROUND

Many insect herbivores who feed on agricultural crops show a remarkable ability to adapt to modern agroecosystems (Crow 1957). Given that some of the most remarkable cases of rapid evolution involve insect herbivores in agriculture, they are ideal species for deeper inquiry on the mechanisms of rapid evolution (Chen and Schoville 2018). Within highly managed agroecosystems, insect herbivores are regularly exposed to novel stressors, such as insecticides, and often show a remarkable ability to adapt to these chemicals (ffrench-Constant 2014). Since the middle of the 20th century, insects have evolved resistance to hundreds of insecticides (Sparks and Nauen 2015).

Although the evolution of insecticide resistance is widely considered to be inevitable for insect herbivores exposed to insecticides in agroecosystems (Gould, Brown, and Kuzma 2018), how populations are able to rapidly evolve resistance to environmental stresses like insecticide exposure remains poorly understood (Gressel 2011; Oppold and Müller 2017; Gould, Brown, and Kuzma 2018; Hawkins et al. 2019). While the biochemical mechanisms of insecticide resistance have been widely studied (Hemingway et al. 2004), how mutations arise in exposed populations is still poorly understood (Brevik, Lindström, et al. 2018).

Transposable elements (TEs) have been relatively unexplored in their contributions to the rapid evolution of agricultural insects (González et al. 2010). Transposable elements are ubiquitous DNA mobile elements within eukaryotic genomes, and play major roles in both genome architecture and the generation of genetic variation (Chénais et al. 2012). As a result of their insertional and recombinational activities, TEs are viewed as a major contributor to the generation of novel mutations within a genome (Chadha and Sharma 2014). For instance, an estimated 50-80% of all mutation events in the fruit fly, *Drosophila melanogaster*, are caused by TEs (Biémont and Vieira 2006). Increasingly, TEs are thought to generate much of the standing genetic diversity that contributes to rapid evolution (Lin et al. 2007; Cordaux and Batzer 2009; González et al. 2008). However, there is limited understanding as to the level of contemporary activity that TEs have within the genomes of agricultural insects.

Given that TEs have been associated with the ability of species to rapidly adapt to novel selection pressures (González et al. 2010; Schrader et al. 2014; Cridland et al. 2013), they may play a major role in the ability of insects to evolve in response to new

management tactics deployed in modern agroecosystems. Depending upon where they land within genomes, TEs can generate many types of mutations, including alternative splicing, gene disruption and silencing, exonization, and changes in expression (Casacuberta and González 2013; ffrench-Constant, Daborn, and Feyereisen 2006). Indeed, TEs are associated with the evolution of insecticide resistance in a number of cases, including in *Drosophila melanogaster* (Daborn 2002; Mateo, Ullastres, and González 2014), *Anopheles gambiae ((*Nikou, Ranson, and Hemingway 2003), and *Culex quinquefasciatus* (Itokawa et al. 2010).

Exposure to insecticides and the other stressors in agroecosystems may facilitate transposable element-mediated mutation, as stress can induce mobilization and activity of transposable elements, leading to increased variation (Maggert 2019). For example, stress can affect the mobilization of TEs in fungi (Chadha and Sharma 2014), insects (Lancaster et al. 2016), and other eukaryotes (Horváth, Merenciano, and González 2017). In *D. melanogaster*, exposure to heat stress is associated with increased rates of transposable element activation, which appears to be due to interactions between heat shock proteins, RNA, and transposable element suppression (Specchia et al. 2010). One well-characterized example of how TEs may play a role in the evolution of insecticide resistance occurred in *Drosophila melanogaster*, where an Accord transposable element insertion near to a gene associated with detoxification led to insecticide resistance by increasing the expression of that gene (Daborn 2002).

The Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) is an important model for the study of rapid adaptation of insects because it appears to evolve resistance very rapidly compared to other insects (Brevik et al. 2018)

and the genome has been recently sequenced and annotated (Schoville et al. 2018). With a global distribution that encompasses the entire potato-growing area of the Northern Hemisphere (Weber 2003), the beetle has been adapted to a wide range of climates (Grapputo et al. 2005; Piiroinen, Lyytinen, and Lindström 2013; Piiroinen et al. 2011), host plants (Izzo et al. 2018, Crossley et al. 2017), and insecticides (Argentine, Clark, and Ferro 1989; Zhu, Lee, and Clark 1996; Alyokhin et al. 2008). Historically, the beetle fed on several plant species in the genus *Solanum* (Jacques 1988), including buffalo bur, *Solanum rostratum*, and expanded its host range to feed on potato, *Solanum tuberosum*, in 1859 in Nebraska (Walsh 1865). After shifting onto potato in the Midwestern US, the beetle soon reached the East Coast (Tower 1906, Gauthier et al. 1981, Hsiao 1985).

Interestingly, different geographic populations of the *L. decemlineata* show different propensities to consume potato and to develop insecticide resistance. Beetles in the Eastern US differ from beetles fromMexico and Plains states in their preference for potato (Izzo et al. 2018), beetles in the Northwest United States evolve resistance slowly (Hawthorne 2020), and beetles in the Eastern United States evolve resistance extremely rapidly. There have been several hypotheses on the geographic origins of the potato-feeding *L. decemlineata* populations, including an endemic origin in the Central Plains of the US (Walsh 1865), northward migration from the highlands of Mexico (Hsiao 1981, Jacobson and Hsiao 1983, Casagrande 1985, 1987, Lu and Lazell 1996), or from hybridization between divergent subpopulations (Hsiao 1985). However, recent work suggests that populations from Mexico and Arizona are substantially divergent from beetles in southern plains of the United States, and that the plains population shifted to feed on potatoes (Izzo et al. 2018). A greater understanding of how ancestral and

descendent populations are related would provide insight on the genomic innovations associated with the beetle's range expansion and association with potatoes, and perhaps, implications for their ability for contemporary evolution. While chemical insecticides remain the major control strategy used to manage this species, the beetle has evolved resistance to every insecticide levelled against it - currently over 55 insecticides (Brevik, Schoville, et al. 2018). However, populations in the Pacific Northwest of the United States and Mexico evolve resistance at significantly lower rates compared to beetles in the Midwest and East Coast of the United States (Dively et al. 2019). It is possible that differences in transposable element content between populations could play a role in these differences (Kofler, Nolte, and Schlotterer 2015; Kidwell and Lisch 1997), and that these differences in TEs may contribute to the ability of *L. decemlineata* to adapt to a wide range of ecological pressures. An assessment of continent-scale transposable element insertions in an insect species that has been exposed to numerous insecticides may provide a broad assessment of such a phenomenon.

In order to understand the role of TEs in evolution, it is important to first characterize the TE community within each genome, to account for the identity of each TE, its specific location within the genome, and relative abundance (Saylor et al. 2013). At the genome level, characterizing community diversity is important because individual transposable elements vary in the ways that they might contribute to evolution, as each family has differences in structural elements, activity levels, and other attributes (Arkhipova 2017). Different types of transposable elements also vary in quantity between different taxonomic groups, and in their molecular behavior in the genome of host species (Tyler et al. 2017). For example, some transposable elements copy themselves throughout

the genome, while others excise themselves and then re-insert at other locations in the genome (Piégu et al. 2015). In order to associate TE activity with evolutionary events, it is important to test how the entire TE community responded to the major evolutionary transitions with the beetle's range expansion. Here we examined 88 resequenced genomes of *L. decemlineata* collected throughout North America (Pelissie et al. *in prep*) to determine how transposable element communities differ between beetle populations. *By aligning the resequenced genomes with the L. decemlineata* reference genome (Schoville et al. 2018), we were able to compare the presence or absence of transposable elements in specific locations across all beetle populations. This is the first study to examine whether the beetle's association with agriculture is related to the level of TE content within resequenced genomes of an agricultural insect herbivore. To measure the diversity of transposable elements in the genome of *L. decemlineata* across sampled beetles, we used the Shannon's H, which takes into account both the presence or absence of each TE in a genome, but also the relative abundance of each type of transposable element. First, we asked if transposable elements are more abundant in the genomes of US beetles compared to Mexican beetles, where the species does not feed on potato and is not considered a pest by humans. Second, we compared transposable element abundance between beetles found on the species' two major host plants (potato *S. tuberosum* vs buffalo burr *S. rostratum*), to determine if the historic host expansion onto potato 160 years ago may have been associated with an increase in TEs(Walsh 1865). Third, to test if there is an association between TEs and insecticide resistance, we tested if transposable element abundance differs between insecticide-susceptible and -resistant beetles,. Fourth, we tested if transposable element abundance differed between beetles in

the Pacific Northwest compared to the rest of the United States, since beetles in the Pacific Northwest evolve resistance less rapidly than other populations in the United States (Dively et al. biorhiv). Finally, we examined whether the insertion locations of transposable elements in the genomes could be used to assess the population structure of this species.

3.3 Results

We found considerable diversity in the composition of transposable elements communities across the 88 *L. decemlineata* genomes. We used two measures of variability: 'non-universal TEs', which are TEs that were not found in *all* beetles, and 'unique TEs', which are TE insertions found in only a *single* beetle included in our study. We detected an average of 8713 (s.d. 7935-9491) non-universal (variable) TE insertions per individual beetle. As for unique TE insertions, we found a wide range within the beetles, with an average of 644 unique TE insertions per beetle, (s.d. ± 1105). However, both measures of variability are highly sensitive to sampling bias, as beetles from populations with multiple sequenced beetles are more likely to share an insertion with closely related beetles, and beetles from a unique location are more likely to have high number of unshared TE insertions. For example, the sole beetle sampled from Arizona contains 7972 unique insertions, while the beetles from Maine, Vermont, New York, and New Jersey (a total of 19 beetles) each have fewer than 152 unique insertions. We found that the beetles contain a relatively narrow range of diversity of TEs, with an average Shannon's H of 5.089, with a range from 5.073 to 5.107.

We found that that overall TE abundance was associated with some, but not all, recent biogeographical and ecological changes in the beetle. We found that beetles collected in the United States had fewer variable TEs than beetles collected in Mexico (t-test, df = 10.81, $P < 1 \times 10^{-6}$) (Figure 1a). On average, beetles collected in the United States hosted an average of 8512 ± 551 variable transposable elements, while beetles collected in Mexico hosted 10198 ± 657 variable transposable elements. We found that beetles collected from *S. rostratum* contained more TEs than beetles collected from potato (*S. tuberosum*) (t-test, df = 29.515, P < 0.005) (Figure 1b). We found that beetle populations classified as either insecticide-*resistant* or -*susceptible* did not differ in the number of variable transposable elements hosted (Figure 1c). Unexpectedly, we found that beetles collected in the Northwest United States contained more TEs than beetles collected in the rest of the United States (t-test, df = 34.609, P < 0.01) (Figure 1d). The diversity and composition of transposable elements, as measured by Shannon's H, did not differ among the populations.

To determine if differences in transposable element insertion locations differentiated populations of *L. decemlineata* in North America, we conducted a Principal Components Analysis using all of the non-universal transposable element insertions of the 334 putatively active TEs, which were TEs with intact protein coding regions . The signal matched our expectations, with populations distinguished based on their known biogeography (Izzo et al. 2018, Pelissie et. al, *in prep*). The major differentiation shown by PC1 and PC2 separated the lowland Mexican population (Jalisco, Oaxaca, Guerrero, Morelos) and part of the population from Maine from the remainder of North American beetles (Figure 5a). PC3 and 4 show separation between the highland Mexican

population, the population from New Jersey (which was reared in a lab for many years, and likely highly inbred) and beetles from the Northwest United States, spread out along a gradient concordant with geography (Figure 2b), each separated from the remainder of North American beetles.

In order to identify which individual transposable element insertions contributed most to differences between populations, we performed a Discriminant Analysis of Principal Components. We found that 21 TEs discriminated most between populations (Table 1). These TEs belong to either the LINE or Mariner groups of transposable elements and are relatively common in the beetle's genome (Schoville et al. 2018). In order to determine if the TEs fell near to genes associated with insecticide resistance, we looked at the scaffolds where the TEs were found, to compare with nearby genes or regulatory regions. However, the scaffolds containing the 21 influential TEs did not contain any predicted genes, so we were unable to determine if there were interactions between these TEs and genes at any distance. This suggests that these TEs are found a) in non-coding regions, b) near unknown genes, or c) that the fragmentation of the genome is impeding our ability to detect these interactions.

3.4 Discussion

We studied the content of TEs in the *L. decemlineata* genome across North America to test for an association between TE activity and current biogeography, and to explore the role of transposable elements in evolutionary transitions. Overall, we found that genome-wide diversity of transposable elements, as measured by Shannon's H, were not significantly associated with major evolutionary transitions in *L. decemlineata*.

However, we found that geographic and host plant expansion of *L. decemlineata* have been coupled with a decrease in total TE abundance. Therefore, we find evidence that historical evolutionary transitions, such as geographic and host expansions, were associated with increased numbers of TEs. This was contrary to our expectation that changes in food source and novel habitats, which may be stressful, would have led to a proliferation of transposable elements in genomes.

The greater number of transposable elements insertion locations found in beetles collected in Mexico may be due to historical divergence between beetles found in the United States and Mexico. With a divergence approximately one million years ago (Izzo et al. 2018), the differences may be due to population drift between these groups over a long timescale. Transposable elements can accumulate in the genomes of organisms for many reasons, including environmental stress (Capy et al. 2000) or the introduction of a novel TE into the genome (Kofler et al. 2018). In Mexico, *L. decemlineata* feeds on more ephemeral plant populations than in the United States, and this difference could play a role in the different numbers of transposable elements found in the two populations – for example, some TEs can have increased activity during droughts (Pekmezci, Karakülah, and Unver 2017), which may have occurred with differing drought frequency in Mexico and the United States. Given that different lineages of species can vary in the accumulation of TEs (Sessegolo, Burlet, and Haudry 2016), subpopulations of species could also vary in their ability to suppress TE proliferation. Therefore, environmental factors or lineage specific ability to suppress TEs could interact to influence the accumulation of TEs along divergent lineages.

The higher number of transposable elements found on beetles feeding on *S. rostratum* compared to those feeding on potato may be indicative of the transition to feeding on potato during the 1850s, when a subset of the beetle population expanded its host range to include potatoes. Presumably a large portion of the beetles feeding on buffalobur did not make this transition, suggesting that many of the individuals with specific transposable element insertions did not make the transition to potato feeding. It is less clear why beetles in the Northwest would have more unique transposable element insertions per beetle, especially since these beetles exhibit lower levels of insecticide resistance. We did not find that beetles which differed in insecticide resistance status differed in the number of unique transposable elements their genomes contain. These two results suggest that population-level exposure to insecticides is not associated with large-scale changes in transposable element content. Instead, specific transposable element insertions may play a larger role than TE content overall. Together, our results suggest that patterns of transposable elements within genomes are detectable at a continental scale, and that they may capture past demographic events and current biogeography.

We found that populations of *L. decemlineata* were differentiated using a principal components analysis on the presence/absence of all transposable element insertion sites in the genomes of these 88 individuals. Although TEs were variable enough to distinguish between North American populations of *L. decemlineata*, we could not assign a significant role in explaining the variation of adaptability in this species. While this may suggest that new TEs largely accumulate neutrally during genetic divergence (Barrón et al. 2014), it is also likely the large scale of our analysis, coupled with the fragmentation of the reference genome, may have obscured any specific

relationship between transposable elements and adaptability in this species. Furthermore, the sheer abundance or diversity of transposable elements in the genome may not provide a direct measure of adaptive evolution, as transposable element content can vary widely between species (Elliott and Gregory 2015), and so far, specific examples of individual transposable element activity have shown more evidence than overall TE quantity in contributing to evolvability (Daborn 2002). This is in line with our finding that Northwest beetles had the highest number of detected unique TEs but are the slowest to adapt to new insecticides.

Across North America, *L. decemlineata* showed variation in TE insertion locations that surprisingly reflected the population structure found using SNP data (Pélissié et al. in prep). This suggests that transposable element activity over the recent expansion and evolution of this species within human agricultural landscapes has been significant enough to differentiate populations. Our results (Figure 2), separating first beetles from the United States and Mexico, are congruent with the current understanding of the recent evolution of *L. decemlineata*. The beetle populations that feed on potatoes evolved from beetles found in the Great Plains region of the United States (Colorado, Kansas, Nebraska, New Mexico, and Texas) are differentiated from beetles found in Mexico. In fact, the US beetle populations (excluding AZ) and the lowland Mexico population (including AZ) have very different mtDNA haplotypes, suggesting that they may actually be separate species (Izzo et al. 2018). Despite the strong differentiation of US and Mexican beetles with mtDNA haplotype data, beetles from the northern and central highlands Mexico (Chihuahua, Saltillo, Puebla, State of Mexico) grouped more strongly than expected based upon geographic distance. The separations between groups

on PC3 and PC4 (Figure 5b) recapitulate the geographic distribution of the beetle, with separations between populations collected from the Northwest, Central and East Coast, Highland Mexico, Lowland Mexico, and lab-reared beetles from New Jersey. Most transposable elements are found in small numbers of individual beetles, with 65% of transposable element insertion sites found in fewer than 10 individual beetles (Figure 3). Overall, our genome resequencing work indicates that TE mobilization appears to be far more frequent in insects than previously suggested.

We found that the 21 transposable element insertions contributing most to differentiation between populations of *L. decemlineata* belonged to two TE families: Mariner and LINE elements (Table 1). Mariner and LINE transposable elements are common in the *L. decemlineata* genome (Schoville et al. 2018), and are particularly widespread in insects (Robertson 1993; Cridland et al. 2013). Mariner family TEs are DNA transposons (Robillard et al. 2016), which cut and paste themselves within genomes, while LINEs are retrotransposons (Specchia et al. 2017) that copy and paste themselves. Several past studies have looked at the role of specific TE insertions and their association with genes in the emergence of insecticide resistance (ref), and so we examined if any of these 21 insertions were near candidate genes. Unfortunately, the transposable elements were located on scaffolds that lacked gene annotations, so it is unclear if these transposable elements are near unannotated genes that may be significant in rapid evolution or if they may be associated with the evolution of stress resistance. Any specific interactions between genes and TEs that may be relevant for rapid adaptation were not detected in our analysis, possibly due to the fragmented nature of the reference genome (Schoville et al. 2018), which is currently assembled with over 20,000

scaffolds, far from a chromosome level assembly. The high number of scaffolds means that there are more 'breaks' in the genome, so transposable elements and genes that are near each other in the genome would not have been detected in our analysis. This is not surprising, as genomic regions containing repetitive sequences such as TEs are often non-coding, and we expect that our estimate of TE content and ability to detect specific gene-TE interactions will likely increase as genome assemblies improve.

Our results suggest that transposable elements may play a role in the rapid adaptation of insect herbivores in modern agroecosystems. Since the Colorado potato beetle expanded its host range to include potatoes in the middle of the 19th century, the species has expanded its range around the world and developed resistance to dozens of insecticides. While we are unable to pinpoint any specific role of transposable elements in these adaptations, we are able to see that transposable elements have been active in the genome of this species, enough so that different populations can be distinguished by examining where transposable elements occur. While we do not see a global proliferation of transposable elements, certain TE insertions may provide some adaptive advantage to beetles, and future work looking more closely at these specific sites may be fruitful in understanding the role of transposable elements in the rapid evolution of insecticide resistance in insects.

3.5 Methods

We sampled beetles from across North America, using geographically dispersed set of 88 samples was selected to maximize information about genomic differentiation across the range of the beetle, as well as to contrast beetle populations that are resistant

and susceptible to imidacloprid (Izzo et al. 2018; Pélissié et al. *in prep*.). We isolated high quality genomic DNA from beetle thoracic muscle tissue using DNeasy Blood & Tissue kits (Qiagen) and then submitted to the University of Wisconsin-Madison Biotechnology Center. DNA concentration was verified using the Qubit dsDNA HS Assay Kit (Life Technologies, Grand Island, NY) and 1 µg of each sample was sheared using a Covaris M220 Ultrasonicator (Covaris Inc, Woburn, MA, USA) to an average insert size of 550 base pairs (bp). Sizing was verified by Fragment Analyzer (Advanced Analytical Technologies, Inc., Ames, IA, USA). Libraries were prepared according the NEBNext® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) with minor modifications. Quality and quantity of the finished libraries were assessed using the Fragment Analyzer and Qubit® dsDNA HS Assay Kit, respectively. Libraries were standardized to 2µM. Cluster generation was performed using HiSeq PE Cluster Kit v4 cBot kits (Illumina Inc, San Diego, CA, USA). Flow cells were sequenced using paired-end, 125 bp sequencing and HiSeq SBS Kit v4 (250 Cycle) (Illumina Inc.) on a HiSeq2500 sequencer. Images were analyzed using the standard Illumina Pipeline, version 1.8.2. We calculated sequencing effort to yield ~12x average coverage for each of our CPB genomes, a quantity sufficient to identify SNPs with high accuracy (Li et al. 2009). Each sample was demutiplexed prior to downstream analysis.

We prepared the *L. decemlineata* reference genome v1.0 (GCA_000500325.1; Schoville et al. 2018) by creating an index with BWA v0.7.101 (Li & Durbin 2009), generating a FASTA file index with SAMTOOLS v1.3.12 (Li & Durbin 2009) and a sequence dictionary with PICARD's CreateSequenceDictionary v2.2.4. We generated one uBAM file (i.e., unmapped BAM file) per forward-reverse pair of the fastq raw reads

using PICARD's FastqToSam and then marked Illumina adapters with PICARD's MarkIlluminaAdapters. We then reverted BAM files to fastq format with PICARD's SamToFastq, aligned the new fastq files to the reference genome with the BWA-mem algorithm and merged all alignments into one BAM file per sample with PICARD's MergeBamAlignment tool. We used mapped BAM files to mark PCR and optical duplicates using PICARD's MarkDuplicates tool. Some of our samples were sequenced on multiple sequencer lanes. For these samples, we marked duplicates first at the lane level (i.e., per replicate), then at the sample level (merging duplicates into a unique BAM output). Finally, we realigned reads around INDELS with GATK's RealignerTargetCreator and IndelRealigner tools.

We used the reference genome of *L. decemlineata* (Schoville et al. 2018) to identify transposable elements by using three programs: 1) RepeatMasker (version 4.0.5) (Smit, AFA, Hubley, R & Green 2015) (*parameters: -s -pa 18 -gff*), which locates known transposable element locations in a genome 2) RepeatModeler (version 1.0.8) (Smit and Hubley 2008) (using parameters *-dir* Custom *-pa 20*), which identifies de-novo repeat elements, and 3) literature searches to identify beetle transposons that were not found within Repbase (Arkhipova et al. 2012), in order to increase our coverage of beetle-specific transposable elements and ensure they were counted among our results, since many of the existing libraries do not contain large numbers of beetle-specific transposable elements. In order to focus on TEs that are most likely to be able to transpose, rather than inactive copies, all TE candidate models found using these three methods were translated and scanned for active protein domains from the Pfam (https://pfam.xfam.org/) database, which allowed the removal of false positives and

highly degraded copies of transposable elements lacking identifiable coding regions. As a result, we generated a list of 334 TEs that maintain functional protein-coding regions and presumably are able to transpose. The curated 334 TEs include 191 LINEs, 99 DNA transposable elements, 38 LTRs, and 5 Helitrons, which was previously reported in Schoville et al. (2018). We then used Repeatmasker (*parameters: -s -pa 18 -gff*) to detect the locations of these 334 'active' transposable elements in the genomes of each of the 88 resequenced *L. decemlineata* collected from around the United States and Mexico, as reported in Pélissié et al. (in prep). This method allowed us to discover the locations of transposable elements within the genome of each beetle individually, providing both an overall count of transposable elements, as well as the specific locations in the genome where copies of each transposable element were found. We generated a presence/absence matrix for each of these sites for all transposable element locations found within any of the 88 beetles, which was used for subsequent analysis. In order to focus on the differences between populations, transposable elements that were shared between all individual beetles were omitted from our analyses. As a result, we generated a matrix of all *non-universal* transposable element insertions, which was any transposable element insertion that was absent from at least one beetle and not fixed for all sampled beetles.

To assess differences in the numbers of transposable elements between beetle populations (United States vs Mexico, *S. tuberosum* vs *S. rostratum* host plants, and insecticide resistant vs. susceptible), we conducted a series of t-tests comparing the total number of non-universal transposable elements found in each group (determined by the relevant comparison, see above) using the R package 'stats' version 3.5.1 (*function: t.test, parameters: (alternative = c("two.sided"), mu = 0, paired = FALSE, var.equal = FALSE,*

conf.level = 0.95) (R Core Team 2018). To assess the diversity of transposable elements within each beetle's genome, we calculated the Shannon Diversity Index (Shannon's H) for each beetle, which allows us to compare the level of TE diversity between beetles. To calculate the Shannon's H Index of each beetle, we used the *diversity* function within the R package *vegan* (version 2.5-3, parameters: *diversity(x, index = "shannon", MARGIN = 1, base = exp(1)*) on the presence/absence matrix of transposable element insertion locations within each genome, followed by an ANOVA (function *aov,* R package *stats*, version 3.5.1 (R Core Team 2018)) to determine differences between groups.

In order to determine differences in transposable elements unique insertion sites between populations of *L. decemlineata*, we analyzed the transposable element location data using a Principal Components Analysis using the presence/absence matrix for each TE location (function *prcomp,* R package *stats*, version 3.5.1 (R Core Team 2018). The data used for this analysis was a presence/absence matrix of all non-universal transposable element insertion locations, that is, all of the unique locations in each genome where a transposable element was found. The function 'prcomp' with settings (*prcomp(x, retx = TRUE, center = TRUE, scale. = FALSE, tol = NULL, rank. = NULL)*) was applied to this matrix to obtain principal components, which were then plotted in ggplot (version 3.1.0). Finally, we applied a discriminant analysis of principal components (DAPC) using the R package *adegenet* (version 2.1.1) to detect which transposable elements contributed the most in distinguishing between populations. This method was used to determine which transposable element locations contributed most to the principal components separating each group (Maine, Lowland Mexico, Highland Mexico, New Jersey, Northwest USA), and was run separately for each group

comparison, each time using these parameters: *dapc(x, grp, n.pca=NULL, n.da=NULL, center=TRUE, scale=FALSE, var.contrib=TRUE, var.loadings=FALSE, pca.info=TRUE, pca.select=c("nbEig","percVar"), perc.pca=NULL, ..., dudi=NULL).* The results of this analysis are shown in Table 1.

To measure the diversity of transposable elements in each resequenced *L.decemlineata* genome, we used the the Shannon's H, which takes into account both the presence or absence of each TE in a genome, but also the relative abundance of each type of transposable element. This metric was developed for use in ecological studies, and takes into account both the abundance of transposable elements (which type of transposable elements are in a genome) but also the evenness of the transposable elements (the count of each type of transposable elements and the differences in counts between the most and least abundant transposable elements). Therefore, the Shannon's H can distinguish between a genome with 10,000 of one type of TE and 10 each of six more types of TE and a genome with 7 types of TE, all with several thousand individual insertion locations. While both genomes have the same representative TEs, the latter is more diverse as it is not dominated by one type of TE.

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3.7 Figures

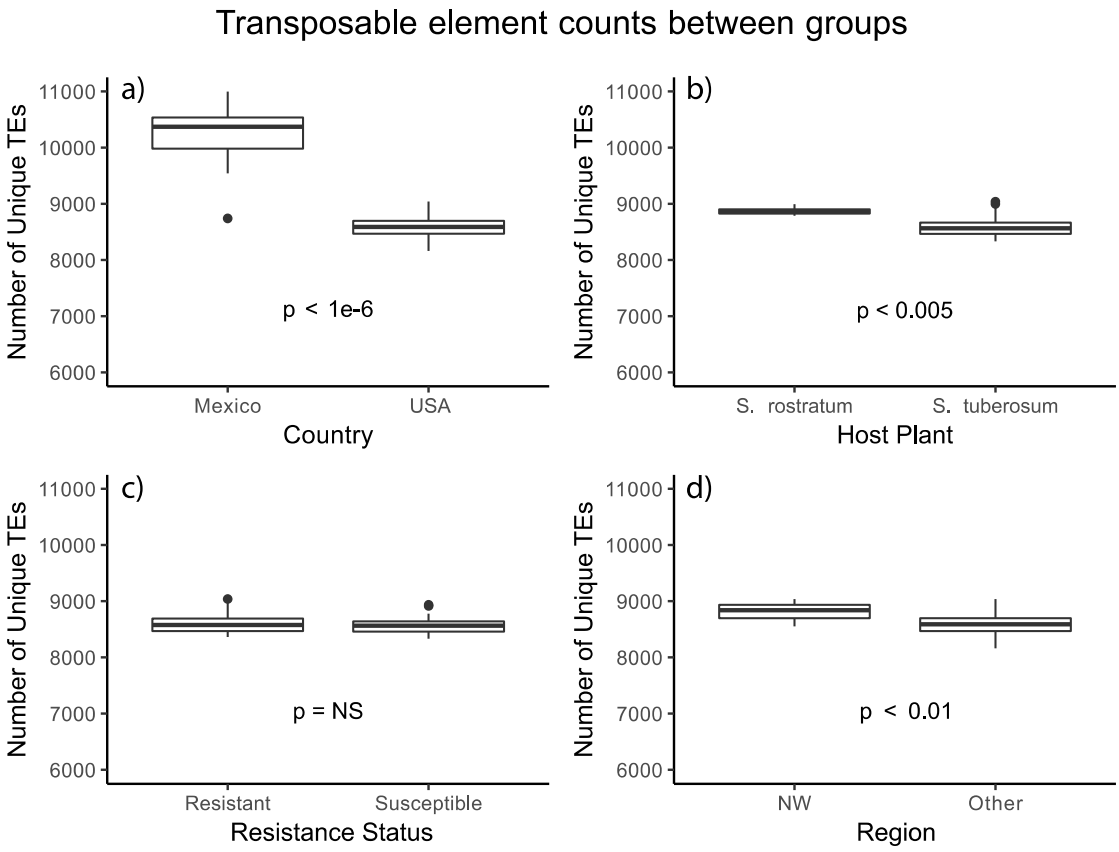

Figure 3.1: Figure 0.3: Comparisons of transposable elements between relevant groups of beetles. a) Beetles collected in Mexico contain more Transposable Elements (10198 ± 65) than beetles collected in the United States (8512 ± 551). b) Beetles found on Solanum rostratum (buffalobur) contain more transposable elements than beeltes collected on Solanum tuberosum (potato). c) Transposable Element counts do not vary depending on resistance or susceptibility to insecticides. d) Beetles collected in the Pacific Northwest host more transposable elements than beetles elsewhere in the United States

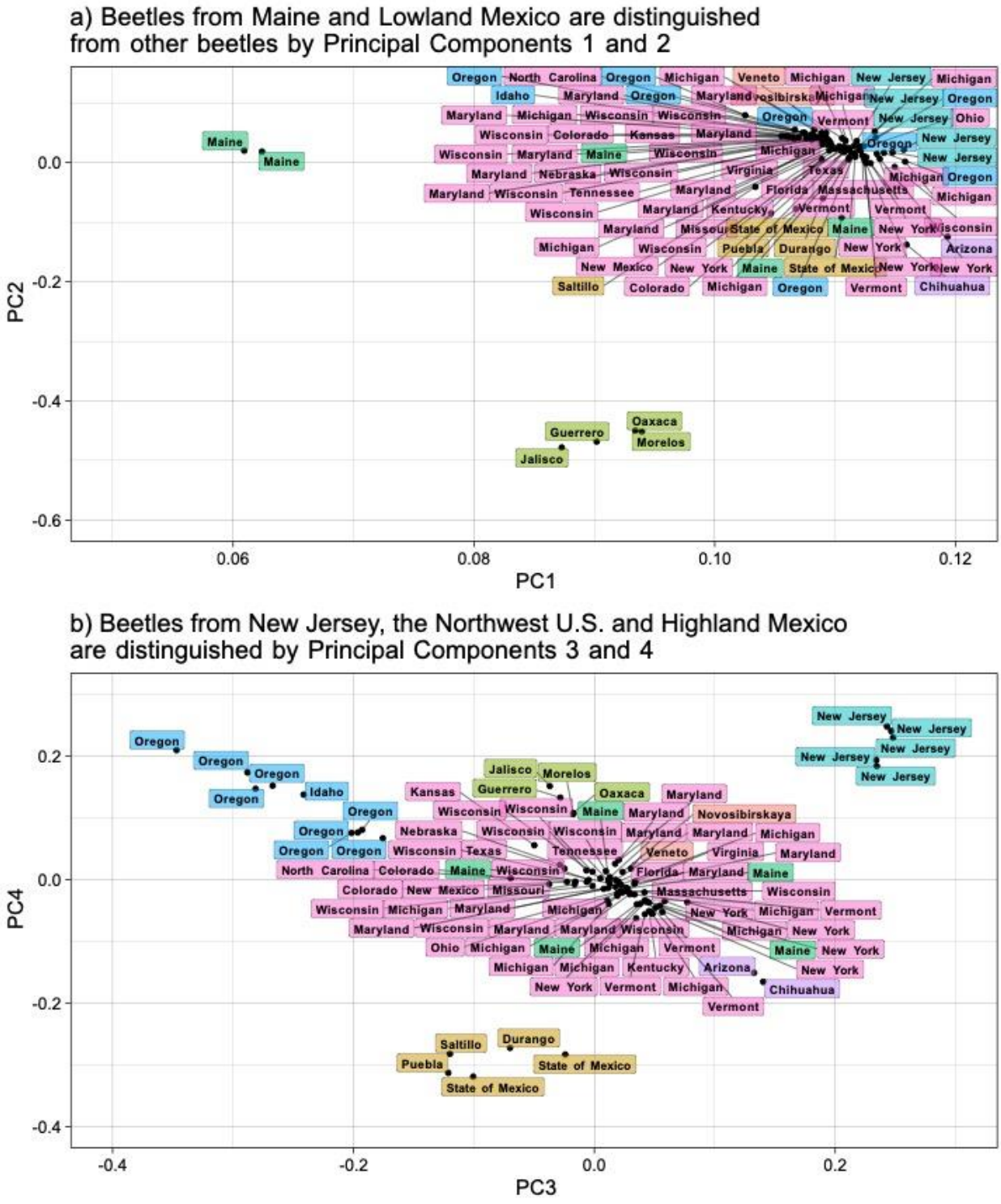

Figure 3.2: Principal Component Analysis of Transposable Elements distinguishes North American populations of L. decemlineata. PC1: 40% of variation, PC2: 2.77% of variation, PC3: 1.6% of variation, PC4: 1.4% of variation. Colors represent the major regions.

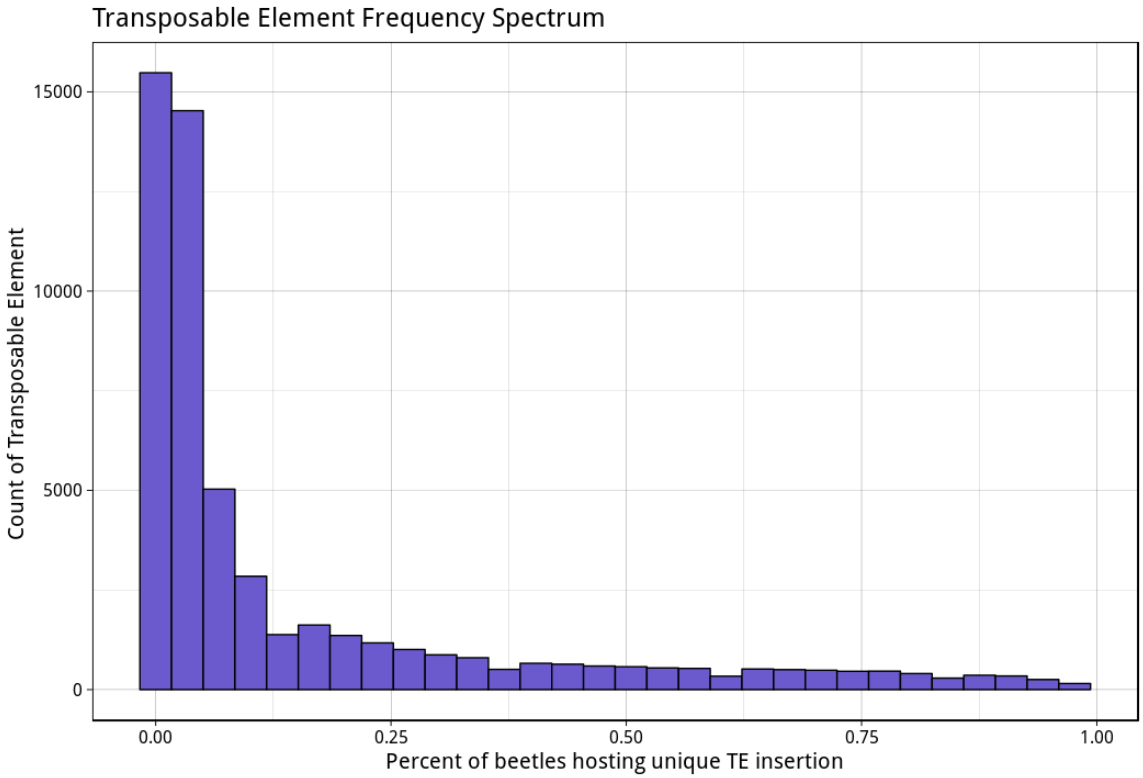

Figure 3.3: Frequency spectrum of Transposable Element insertions across all beetles. Most transposable elements are present in few beetles.

3.8 Tables

Table 1: The transposable element insertions with the highest loadings contributing to distinguishing between populations of L. decemlineata, according to DAPC

Beetle Group	Transposable Element Insertions Contributing to Discrimination Between Groups	Scaffold
Southwest Mexico	TcMar-Tc1	22415
	LINE/Penelope	22934
	TcMar-M44	21376
	TcMar-M44	11017
	LINE/L2	14189
	LINE/CR1	10723

	LINE/L2 LINE/L2 LTR/Copia LINE/L2 LINE/Penelope	12090 13361 12313 17472 17453
Maine	LTR/Gypsy LINE/Penelope	22303 19051
New Jersey	LINE/Penelope	18896
Northeast Mexico	LINE/Penelope TcMar-Mariner LINE/Penelope DNA/TcMar-Tc1	21875 24184 15506 12493
Northwest United States	DNA/TcMar-Tc1 LINE/L2 LINE/Tad1	19767 20304 19976

CHAPTER 4: EFFECTS OF IMIDACLOPRID ON TRANSGENERATIONALLY INHERITED METHYLATION IN THE COLORADO POTATO BEETLE, LEPTINOTARSA DECEMLINEATA

4.1 Abstract

Insecticide use is pervasive as a selective force in modern agroecosystems. Insect herbivores exposed to these insecticides have been able to rapidly evolve resistance to them, but how they are able to do so is poorly understood. One possible but poorly explored explanation is that exposure to sublethal doses of insecticides may alter epigenetic patterns that are heritable. For instance, epigenetic mechanisms, such as DNA methylation that modify gene expression without changing the underlying genetic code, may facilitate the emergence of resistant phenotypes in complex ways. We assessed the effects of insecticide exposure on DNA methylation in the Colorado potato beetle, *Leptinotarsa decemlineata,* examining both global changes in DNA methylation and specific changes found within genes and transposable elements. We found that exposure to insecticide led to decreases in global DNA methylation for parent and F_2 generations, and that many of the sites of changes in methylation are found within genes associated with insecticide resistance, such as cytochrome P450s, or within transposable elements. Exposure to sublethal doses of insecticide caused heritable changes in DNA methylation in an agricultural insect herbivore. Therefore, epigenetics may play a role in insecticide resistance, highlighting a fundamental mechanism of evolution while informing how we might better coexist with insect species in agroecosystems.

4.2 Introduction

Insect herbivores in agroecosystems show a remarkable ability to rapidly adapt to novel forms of environmental stress, including synthetic insecticides (Brevik et al. 2018a). Current data suggest that at least six hundred arthropod species have developed resistance to over three hundred insecticidal active ingredients, with tens of thousands of reports of resistance worldwide, the vast majority since 1945 (Whalon et al. 2012). While it is often considered inevitable that insects will evolve resistance to insecticides (Alyokhin et al. 2015; Gould et al. 2018a), how insect populations rapidly evolve this resistance remains poorly understood (Gressel 2011; Oppold and Müller 2017a; Gould et al. 2018b). Current evolutionary theory falls short of explaining the rapid evolution of insecticide resistance for multiple reasons (Laland et al. 2014). First, insect populations are unlikely to possess the standing variation to provide advantageous mutations to novel insecticidal toxins (Carrière and Tabashnik 2001). Secondly, new mutations may occur too infrequently to drive the pace of insecticide resistance (Karasov et al. 2010; Keightley et al. 2015), and the same insect species are repeatedly the first ones to develop resistance to new insecticides when they are introduced (Brevik et al. 2018a). If rates of insecticide resistance are based solely on our expectations of traditional Darwinian evolution, then repeated effects of extreme bottlenecks and low mutation rates should limit the ability for insects to develop resistance (Sax and Brown 2000). The paradox of insecticide resistance evolution is that despite experiencing strong selection that reduces insect population size and genetic variation, insects are still able to rapidly and repeatedly adapt.

Insecticide resistance occurs with the emergence of resistant phenotypes that are able to tolerate increasingly higher concentrations of insecticide. Much of our current understanding of insecticide resistance focuses on two major types of genetic mechanisms: *qualitative* changes, where mutations at a gene target site cause an insecticide to be less effective, and *quantitative* changes, such as increases in gene transcription that enhance the production of metabolic enzymes or increase the rate of toxin excretion due to accelerated metabolic pathways (Bass and Field 2011; Ffrench-Constant 2013; Liu 2015). While much of insecticide resistance literature has focused on qualitative changes because they are more straightforward to detect, quantitative changes in the expression of detoxification genes have been more important in conferring broad spectrum resistance (Li et al. 2007; Perry et al. 2011; Cui et al. 2015). Multiple studies have demonstrated how increased transcription of detoxification genes, such as P450s, glutathione-S-transferases, and esterases, underlie insecticide resistance (Perry et al. 2011; Ffrench-Constant 2014; Liu et al. 2015). In addition, researchers have observed that while insecticide resistance often increases in response to the frequency of insecticide use (Malekmohammadi 2014; Yang et al. 2014; Tang et al. 2015), it is easily lost when insecticides are discontinued (Ffrench-Constant et al. 1988; Foster et al. 2000). This phenotypic plasticity in response to changing environmental conditions may be due to epigenetic changes, which are able to change more rapidly than DNA sequence changes (Roberts and Gavery 2012).

Epigenetics is the study of modifications that change how genes are expressed without changing the underlying DNA sequence of an organism. DNA methylation is a well-documented mechanism of epigenetic inheritance that can influence phenotypic

variation (Mendizabal et al. 2014). Methylation is the addition of a methyl group (CH_3) to the 5-carbon position of cytosines at CpG sites (Flores et al. 2013), which alters the level at which genes are transcribed without altering the underlying DNA sequence. DNA methylation is widespread in insects (Glastad et al. 2011; Thomas et al. 2020), including beetles (Snell-Rood et al. 2013; Feliciello et al. 2015; Cunningham et al. 2015). Methylation can occur throughout the genome, though its function may differ based on where it is located. In insects, the genomic regions that tend to exhibit DNA methylation are usually *within* genes and coding regions (Hunt et al. 2013a), while promoter regions remain largely clear of methylation. Increases in intragenic methylation in insect genes is associated with increased expression of those genes, as well as an increase in the number of alternative splice variants (Flores et al. 2012). When DNA methylation occurs in promoter regions, it is associated with gene silencing, as the methyl groups interfere with transcription machinery (Hunt et al. 2013b).

Emerging evidence suggests that insecticide exposure can directly and indirectly drive the evolution of insecticide resistance in agroecosystems via epigenetic processes (Brevik et al. 2018a). Pesticides may directly stimulate the expression of advantageous phenotypes, which may be underwritten by epigenetic modifications. Continued insecticide exposure in populations developing resistance would thus operate as 'natural selection' and selectively increase the frequency of insect phenotypes that are adaptive to pesticides. Changes in the DNA methylation state of genes have been associated with insecticide resistance, and may be "a sensitive and reactive mode of action to enhance early-on adaptation" (Oppold and Müller 2017b). For example, the green peach aphid, *Myzus persicae*, can gain insecticide resistance through the duplication of esterase genes

and subsequent overexpression of esterases (Field et al. 1989). However, Field et al. (1989) found that when methylation was lost on these genes, aphids became susceptible again, suggesting that methylation of esterase genes led to increased expression in aphids, and demethylation is associated with gene suppression. Importantly, methylation patterns were maintained over multiple generations, and the increased gene copy number was maintained, so it is possible that resistant aphids that had lost resistance through demethylation could quickly regain resistance through remethylation. In addition, insecticide exposure has been shown to alter patterns of global DNA methylation in bumblebees (Bebane et al. 2019) and honeybees (Paleolog et al. 2020), suggesting that insecticide exposure may interact with DNA methylation, which in turn shapes phenotypic responses to insecticide. Some changes in DNA methylation due to exposure to toxins or demethylating agents appear to be heritable in arthropods (Vandegehuchte et al. 2010; Oppold et al. 2015), but previous research has focused primarily on species such as *Daphnia magna* which reproduce asexually, and it is unclear if these changes persist through sexual reproduction. To date, no previous study has carefully examined how insecticide exposure influences heritable genome-wide epigenetic modifications in an agricultural insect herbivore.

If the epigenetic modifications that respond to environmental stress are heritable, they may play a role in rapid evolutionary change. For example, in the greater wax moth, *Galleria mellonella*, changes in DNA methylation and histone modifications facilitate the evolution of resistance to parasitic fungi by translating selection pressure into a heritable phenotype (Mukherjee et al. 2019). The parasitic wasp *Pimpla turionella* has been shown to modulate the epigenetics of host insects, decreasing DNA methylation, histone

acetylation, and deacetylation, possibly leading to increased survival of larvae within hosts (Özbek et al. 2020). Beyond insects, it is thought that the evolution of finches in the Galapagos was mediated in part by changes in epigenetic marks, with genes associated with beak formation showing epigenetic changes (Skinner et al. 2014). Further inquiries into the role of epigenetics in the evolution of insecticide resistance may provide pathways to understanding the complex phenomenon of rapid evolution.

In addition to direct effects, the interplay of transposable elements and DNA methylation could influence insecticide resistance (Lippman et al. 2004; Xie et al. 2013). One of the primary roles of DNA methylation in eukaryotic genomes is to silence the activity of transposable elements (Zemach et al. 2010), which are mobile genetic elements that can either "jump" within the genome or 'copy-paste' themselves, proliferating throughout the genome (Fablet and Vieira 2011; Göke et al. 2016; Hosaka and Kakutani 2018). TEs play essential roles in the structure and function of the genome, and the relationship is often symbiotic rather than parasitic (Dooner and Weil 2013). TEs are responsible for most mutations within genomes and account for the bulk of the volume of most eukaryotic genomes (Fedoroff 2012). They are also likely responsible for some of the most important structural elements in the genome, such as introns (Huff et al. 2016). TEs generate genetic variation (Kidwell and Lisch 1997) via a number of mechanisms, including inserting upstream of a gene and altering gene expression levels (Daborn 2002) and duplicating genes (Berger et al. 2016), both of which have been implicated in the evolution of insecticide resistance. Changes in the DNA methylation state of TEs can also be associated with rapid evolution, and there is considerable evidence that stress, such as exposure to toxins, can lead to the mobilization of

transposable elements (Chadha and Sharma 2014; Horváth et al. 2017; Cappucci et al. 2019). In insects (*Drosophila melanogaster)*, exposure to heat stress is associated with increased rates of transposable element activation, which appears to be due to interactions between heat shock proteins, RNA, and transposable element suppression (Specchia et al. 2010).

The Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) is an important model for the study of rapid adaptation in insects. The beetle appears to evolve resistance more rapidly compared to other insects (Brevik et al. 2018b). It has evolved resistance to every insecticide used against it, currently over 55 insecticides (Alyokhin et al. 2008). With a global distribution that encompasses the entire potato-growing area of the Northern Hemisphere (Weber 2003), the beetle has adapted to a remarkable range of climates (Lehmann et al. 2014), host plants (Jacques 1988, Crossley et al. 2017), and insecticides (Argentine et al. 1989; Zhu et al. 1996; Alyokhin et al. 2008). Before encountering the potatoes planted by European settlers in what is now the United States, the beetle fed on several plant species in the genus *Solanum*, including buffalo bur, *Solanum rostratum* (Jacques 1988). The beetle was first reported to have expanded its host range to feed on the potato, *Solanum tuberosum*, in 1859 in Nebraska (Walsh 1865). Following its invasion into Europe and continuing into Asia, the beetle has evolved rapidly to face a number of novel stressors and environments, including dozens of insecticides and colder northern climates (Grapputo et al. 2005; Alyokhin et al. 2015). The beetle evolves resistance to new insecticides in an average of 34 generations, or about 10 years (Brevik et al. 2018b). Therefore, the beetle's widespread distribution, adaptability, and impact on potato makes this species ideal for

understanding how the effects of insecticide exposure shape the responses of insect herbivores to the management of agroecosystems.

To determine if exposure to insecticide leads to changes in DNA methylation in the Colorado potato beetle, we used an experimental approach to test whether insecticide exposure altered heritable patterns of DNA methylation in the Colorado potato beetle across multiple generations. By sequencing the DNA epigenome of exposed and F_2 beetles, we tested if the epigenetic responses could be heritable. First, we tested how exposure to a common neonicotinoid insecticide, imidacloprid, influenced patterns in global DNA methylation in the parent and F_2 generations. Our study design allowed us to test for direct effects of imidacloprid on DNA methylation levels on the exposed generation, and whether these patterns persisted through two generations. Second, we tested where differential methylation occurred in the genome, by looking at each site (CpG nucleotide) that was found to be differentially methylated in beetles exposed to insecticide treatments. This analysis examined which differentially methylated sites were associated with a) annotated genes, b) the flanking regions of annotated genes, or c) annotated transposable elements. Together, these analyses provide insight as to how DNA methylation may play a role in the rapid adaptation of the Colorado potato beetle to insecticides.

4.3 Results

All beetles exposed to insecticides (1 ppm imidacloprid, 0.1 ppm imidacloprid, 1 ppm analog) showed a decrease in global DNA methylation compared to the beetles exposed to water. The decrease in global DNA methylation was maintained across two

generations until the F_2 generation (Figure 2, p-values in figure). Overall, global DNA methylation was quite low. On average 0.047% of cytosine nucleotides were methylated per beetle, with a range of 0.029-0.075%. However, exposure to insecticides decreased methylation from 0.06% (0.043-0.075%) in the control to an average of 0.042% (0.029-0.06%) in the treated groups, a difference of approximately 0.02%. There was no effect of beetle generation on global DNA methylation, and beetle generation and treatment did not significantly interact. Because the ANOVA test showed that the three treatments were each significantly different from the water control but not from each other, we compared all three insecticide treatments together with the control in subsequent analyses. Analysis of differential methylation within each treatment verified that variation in differential methylation was smaller within each treatment than between treatments ($F = 282.08$, $p < 0.001$), showing consistent effects across generations.

In comparing the three insecticide treatments with the water control, we found that 221 sites showed differential methylation of 10% or more, using a Q-value cutoff of 0.01 (Figure 3). These values were chosen to select sites with both notable changes in methylation and confidence in our findings. Of the 221 differentially methylated sites, nine of these sites were found within four gene annotations in the genome (Table 1a), with multiple sites per annotation. Two of these genes are cytochrome P450s that are already associated with resistance, *LDEC011287* and *LDEC015052*. *LDEC011287* contained three differentially methylated sites and *LDEC015052* had two. All five sites showed increased levels of methylation. One of the remaining two genes is uncharacterized in the current genome annotation and showed one site of increased methylation and one site of decreased methylation. The fourth gene is a putative cyclin-

dependent kinase, and both methylation sites found within this gene showed decreased methylation. Among the remaining differentially methylated sites, three were found within the 2 kb flanking regions of annotated genes (Table 1b). Two of these sites occurred within the flanking region of the same putative cyclin-dependent kinase, *LDEC015089*, and the third occurred within the flanking region of a glycoside hydrolase. Close to 39% (86) of the differentially methylated sites fell within 47 transposable element annotations, with some transposable elements containing multiple variable methylation sites (Table 1c). A Chi-square test shows that differentially methylated sites were overrepresented in transposable elements compared to the genome as a whole (Chi-square = 5.6365, *p*-value < 0.05). Most of these transposable elements were LINE elements, though a number of other types are also represented.

Although beetles exposed to imidacloprid showed a decrease in global methylation, the location of the individual methylation sites varied by treatment. When each analysis was independently compared to the water control, we found that only 1.55% or 13 sites showed a similar pattern in differential methylation across all three treatments (Figure 4). While none of these 13 sites overlapped with any gene annotations in the genome or with any flanking regions for gene annotations, three of them were found within LINE transposable element annotations (Table 2).

4.4 Discussion

The emerging perspective in environmental epigenetics is that environmental exposure to a range of chemicals can cause lasting heritable effects. Environmentally-

induced changes in epigenetics can lead to a number of phenotypic effects that persist through generations, from disease etiology (Nilsson et al. 2018), to adaptive responses to environmental change (Thiebaut et al. 2019). These epigenetic changes can influence developmental bias, phenotypic plasticity, and niche construction, contributing to evolutionary dynamics (Jeremias et al. 2018). Indeed, it is thought that environmentally-induced changes in epigenetics may have contributed to the evolution and diversification of Darwin's finches (Skinner et al. 2014). DNA methylation has been shown be heritable across multiple generations, which may lead to sustained adaptation. Recent research has shown that DNA methylation influences critical patterns of gene expression in insects as well (Glastad et al. 2014). In social insects, gene expression modulated by DNA methylation plays a role in the determining of caste (Glastad et al. 2011; Weiner et al. 2013), while in other species, changes in DNA methylation are associated with changes in sensitivity to toxic chemicals (Field et al. 1989; Oppold et al. 2015). Insight into these mechanisms provides novel ways of understanding the rapid emergence of insecticide resistance in insects and may help to resolve the paradox of insecticide resistance.

We show that insecticide exposure can influence the patterning of heritable epigenetic modifications in the Colorado potato beetle. Exposure to insecticides decreased global methylation in the beetle, highlighting a possible apparent trade-off between detoxification and epigenetic regulation. Previous work has shown that toxin exposure may reduce global DNA methylation (Hunter et al. 2014; Oppold et al. 2015), and one possible mechanism is due to competition between biochemical pathways. DNA methylation of genomic DNA is dependent upon the availability of methyl groups and S-adenosylmethionine (Lee et al. 2009). S-adenosylmethionine provides the methyl groups

for methyltransferases to methylate DNA. Glutathione, which is an antioxidant that conjugates with xenobiotic toxins requires homocysteine, which is also needed as a precursor for S-adenosylmethionine (Enayati et al. 2005). In the presence of toxins, detoxification becomes imperative and depletes homocysteine (Oppold and Müller 2017b), which may lead to a lack of S-adenosylmethionine available for DNA methylation and a corresponding decrease in DNA methylation in the genome (Lee et al. 2009; Oppold and Müller 2017b). In this case, it could be that the biochemical pathways that are involved in detoxification are depleting the biochemical precursors that are needed to methylate DNA. Given the minimal overlap across treatments in the differentially methylated cytosines, our data suggests that changes in DNA methylation may occur randomly within the genome.

Interestingly, we did not find a clear relationship between insecticide toxicity and global DNA methylation. Despite the reported lack of insecticidal activity for the imidacloprid analog (Kagabu et al. 2007), it caused similar changes in global DNA methylation as the more toxic imidacloprid. Furthermore, even the 0.1 ppm dosage caused a similar effect. The parallel responses across all insecticide treatments suggest that acute toxicity may not be as important as mere exposure to novel compounds. Additionally, all treatment doses led to a similar decrease in global DNA methylation, suggesting that very low doses (much lower than many insects receive in the field) may play a role in causing changes in methylation (Desneux et al. 2007). Therefore as suggested by Lee et al. (2009), simply the exposure to novel chemicals may cause long lasting and unpredictable effects within the genomes of exposed individuals.

The specific genes where DNA methylation changed provide support for a role of methylation in insecticide resistance. Exposure to imidacloprid increased methylation of cytochrome P450s, which is one of the main groups of enzymes associated with detoxifying insecticides in insects (Feyereisen 1999; Scott 1999; Puinean et al. 2010; Liu et al. 2015). Some examples of insecticide resistance in the Colorado potato beetle are due to either mutations in cytochrome P450 genes or in changes in the levels of transcription of these genes (Clements et al. 2016). Glycoside hydrolases are genes involved in the breakdown of glycoside which are compounds found in plants, and are found only in *Phytophaga* (leaf-eating beetles) among insects (Busch et al. 2019), a clade of plant-eating beetles, which includes the *L. decemlineata*. This may be significant because many of the genes that have evolved to deal with plant toxins are able to be used by the beetle to adapt to the toxins found in insecticides (Zhu et al. 2016). The downregulation of cyclin-dependent kinases may be more challenging to understand, because these genes are involved in regulating the cell cycle (Malumbres 2014), though it is notable that this is the one type of gene that showed changes in methylation in both the gene and in neighboring flanking regions. Together, the narrow subset of genes that showed changes in DNA methylation levels is surprising, and further inquiry on these and similar genes may yield insight into how these genes are expressed and how changes in DNA methylation influence beetle phenotypes.

It is remarkable that among the 221 sites that showed changes in DNA methylation, many fell within transposable elements. Given that approximately 17% of the genome is made of up TEs (Schoville et al. 2018) but 39% percent of differentially methylated sites from this study are found within TEs, it appears that TEs may be subject

to a disproportionate amount of differential methylation. While the overrepresentation of TEs as sites for differential DNA methylation could be influenced by the assembly of the reference genome, it may also be possible DNA methylation within transposable elements can be associated with exposure to insecticide. Transposable elements are commonly suppressed and prevented from causing mutation by DNA methylation (Lippman et al. 2004). If insecticide exposure alters the DNA methylation of transposable elements, they may be more able to generate mutation in an affected insect, and these mutations may be associated with resistance. Indeed, in *D. melanogaster*, repeated insertions of transposable elements within stress-response genes may be associated with increased stress tolerance (Merenciano et al. 2016). Our results lend support to a pathway by which changes in genome regulation may drive a dynamic interplay between epigenetics and transposable elements, which may be contribute to the development of insecticide resistance.

Our study was limited for several reasons. We did not track the pedigree of each exposed beetle, but instead looked at colony-wide effects, which limited our ability to assess the maintenance of DNA methylation changes at specific sites. We also did not link changes in methylation to either gene expression or phenotypic changes, which would have provided a more robust assessment of how changes in DNA methylation due to insecticide exposure impact the fitness of beetles when encountering insecticides or other stressors. Nevertheless, we provide initial confirmation of the presence of DNA methylation in the Colorado potato beetle and how insecticide exposure causes changes in methylation in genes associated with resistance. In addition, we show that these

changes in DNA methylation can last for at least two generations, indicating how epigenetic variation can be heritable within a population.

We suggest that complex interactions between insecticide exposure, transposable element activity, and epigenetics may play a role in insecticide resistance. These elements together may contribute to the ability of insects to rapidly evolve in agroecosystems by explaining how our expectations surrounding bottlenecks, low mutation rates, and strong selection do not always line up with the rate of evolution of insecticide resistance. Further research incorporating more analyses are necessary to validate these results - including transcriptome sequencing and phenotypic assays to determine if changes in DNA methylation are associated with changes in transcription and insecticide resistance. Future research may also choose to focus on specific genes, such as cytochrome P450s, s to more fully assess and understand the nuances of how changes in DNA methylation influence the genes associated with insecticide resistance and other stressors. Our results provide a strong imperative for comprehensive, multigenerational longitudinal studies that follow populations of insects after insecticide exposure, monitoring epigenetic changes, gene expression changes (including transposable element expression), and whole genome sequencing to determine how these aspects of evolution are entangled over time.

4.5 Methods

4.5.1 Insect Rearing

We started a beetle colony by collecting 50 adult beetles from three organic potato farms in Vermont in June 2015 and pooling them into a single colony. We chose

to use imidacloprid, a neonicotinoid insecticide, because it is the most widely used insecticide currently deployed against the beetle (Mota-Sanchez et al. 2006). In order to minimize the possibility that that the collected beetle populations had been previously exposed to imidacloprid, we carefully selected farms that have been certified organic since the early 1990s, before the introduction of imidacloprid. We reasoned that prior exposure of a beetle population to imidacloprid may have been selected for higher overall resistance, which may influence epigenetic responses in this study. However, organic growers are allowed to use spinosad to conform to organic standards, which shows a low to moderate cross-resistance with imidacloprid (Mota-Sanchez et al. 2006). Therefore, the field-collected beetles likely have a low to moderate level of prior resistance to imidacloprid. In order to minimize any maternal effects arising from previous environmental exposure, the colony was reared for four generations before the experiment took place. We maintained the beetle colonies on live potato plants at 24°C (16:8 LD) in 60 cm x 60 cm x 40 cm cages using potato plants. The potato plants (*Solanum tuberosum* L., var. Kennebec) were in Fafard 3B potting mix (Fafard, Agawam, MA, USA) in 10.2 cm pots in the greenhouse for 6-8 weeks. Plants were fertilized with a liquid fertilizer twice a week during watering (17-4-17, N-P-K). Plants were grown for 6-8 weeks before they were fed to the beetles. Eggs were removed from each colony twice a day and moved to smaller rearing cages to minimize cannibalism and prevent overlap of generations.

4.5.2 Study Design

To determine if insecticide exposure changed DNA methylation patterns in the Colorado potato beetle, we exposed beetles to sublethal dosages of the neonicotinoid insecticide imidacloprid. We sampled adult beetles from each treatment during the exposed and F_2 generations, and sequenced the beetles using a whole genome bisulfite sequencing (WGBS) approach to assess changes in DNA methylation throughout the genome. Given that environmental conditions are thought to influence patterns of DNA methylation independent of ancestry, we elected to use a mass rearing approach of selecting individuals from a colony, rather than following a pedigree breeding approach to test for the possibility of intergenerational (F_2) inheritance. By selecting random individuals from the colony, we used a more conservative approach by incorporating greater level of heterogeneity, allowing us to detect whether the patterns of DNA methylation were similar across beetle individuals from the same treatment, regardless of ancestry.

4.5.3 Treatments

We developed insecticide treatments that would impose different levels of stress. The four treatments varied in their dosage and toxicity (1 ppm imidacloprid, 0.1 ppm imidacloprid, 1 ppm imidacloprid analog, and water control). We first calculated the LD_{10} dosage by determining the dosage that caused 10% of the exposed beetles to die. We calculated that the LD_{10} was at 1 ppm dosage, which would deliver a stressful, yet sublethal, dose. Even though all concentrations below the LD_{50} level are considered to be sublethal, the 0.1 ppm treatment was intended to be fully sublethal to all beetles (Olson et al. 2000).

In order to control for chemistry of the compound itself, we used an imidacloprid analog. Analogous to cage-controls in ecological experiments, the imidacloprid analog was a compound modified to be less toxic than imidacloprid, which could allow us to separate the effects of the compound on DNA methylation from the degree of toxicity. The imidacloprid analog was custom synthesized to be chemically similar to imidacloprid, but with very little insecticidal activity due to a slight difference in molecular structure (Kagabu et al. 2007). Figure 1 shows how the molecular structure of imidacloprid and the imidacloprid analog differ, where imidacloprid has a hydrogen, the analog contains a methoxycarbonyl group (-COOMe). We applied the insecticides on fourth instar larvae selected from the colony. For each treatment, a 1 µl droplet of treatment solution was applied to the dorsal side of the thorax of 50 fourth-instar larvae. After the exposure to each treatment, the surviving larvae from each treatment were used to found separate colonies that propagated over four additional generations. For genome sequencing, we sampled the adults from each treatment for four subsequent generations, including the exposed generation. Due to budget limitations, only the exposed and F_2 generations were included in this study.

4.5.4 DNA Extraction and Sequencing

In both the exposed and F_2 generation, bisulfite sequencing was conducted on the DNA extracted from 16 exposed beetles, 4 from each treatment, for a total of 32 individuals. We extracted DNA from half of the thorax and abdomen of adult beetles for genomic DNA sequencing using the Omega Bio-Tek E. Z. N. A. Mollusc DNA kit (Omega Bio-tek, Norcross, GA). Following genomic DNA extraction, we verified DNA

quality and concentration using agarose gel electrophoresis and Qubit prior to library construction. After quality testing, positive control DNA was added and the DNA was fragmented into 200-400bp using Covaris S220. Sequencing adapters were ligated to the DNA fragments. DNA libraries were bisulfite treated using EZ DNA Methylation Gold Kit (Zymo Research). Library concentration was quantified by Qubit 2.0, and then was diluted to 1 ng/μl before the insert size was checked on Agilent 2100 and quantified using qPCR. Libraries were then pooled and then paired-end sequencing was conducted via Illumina, with 150 basepair reads.

4.5.5 Analysis

For analysis, we relied on the packages Bismark (Krueger and Andrews 2011) and Methylkit (Akalin et al. 2012) to examine which cytosine nucleotides exhibited differential methylation between treatments. Sequenced reads were checked for quality using FastQC (Andrews 2010), adapters were trimmed and deduplication was done using Bismark and samtools (function *merge*). Sequenced reads were mapped to the *L. decemlineata* reference genome (v. 1.0) using Bismark (*default parameters*). Each site had a mean coverage of 60.75.

To assess differential methylation between treatments, we used the R package Methylkit version 3.11, which provided assessments of which sites exhibited differential methylation (function *processBismarkAln*, parameters: *read.context="CpG", nolap=FALSE, mincov=10, minqual=20* and function *filterByCoverage*, parameters: *lo.count=10,lo.perc=NULL, hi.count=NULL, hi.perc=99.9*). To determine if changes in methylation sites were consistent between generations, we utilized an ANOVA approach

(functions *lm/anova,* from package *stats v3.6.2*) comparing differential methylation within and between treatments. Global DNA methylation was calculated as methylated cytosines as a percentage of all cytosines. For all tests, a minimum change of 10% methylation level with a Q-value cutoff of 0.01 was used (function *getMethylDiff*, parameters: *difference=10, qvalue=0.01*). In order to assess the effect of treatment, generation, and treatment x generation on CpG methylation, we conducted ANOVA tests in R (package *stats v3.6.2,* function *aov*).

In order to find which differentially methylated sites were associated with certain genomic features (gene annotations, 2 kb gene flanking regions, and transposable elements), we used the package bedtools (*v.2.29.2*) functions 'flank' and 'intersect'. Gene annotations were used from the Colorado potato beetle *(*version 1.0) reference genome (Schoville et al. 2018), and transposable elements were annotated using the discovery pipeline described in (Brevik *et al. in prep*) using RepeatModeler (version 1.0.8) (Smit and Hubley 2008) (using parameters *-dir* Custom *-pa 20*), We then used Repeatmasker (*parameters: -s -pa 18 -gff*) to detect the locations of these 334 'active' transposable elements in the genome.

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4.7 Figures

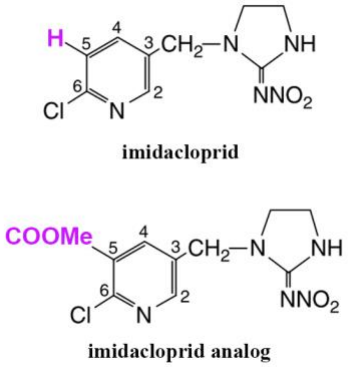

Figure 4.1: Comparison of the chemical structure of imidacloprid with the chemical structure of the imidacloprid analog used in this study, where a hydrogen has been replaced with a methoxycarbonyl group. Differences highlighted in pink.

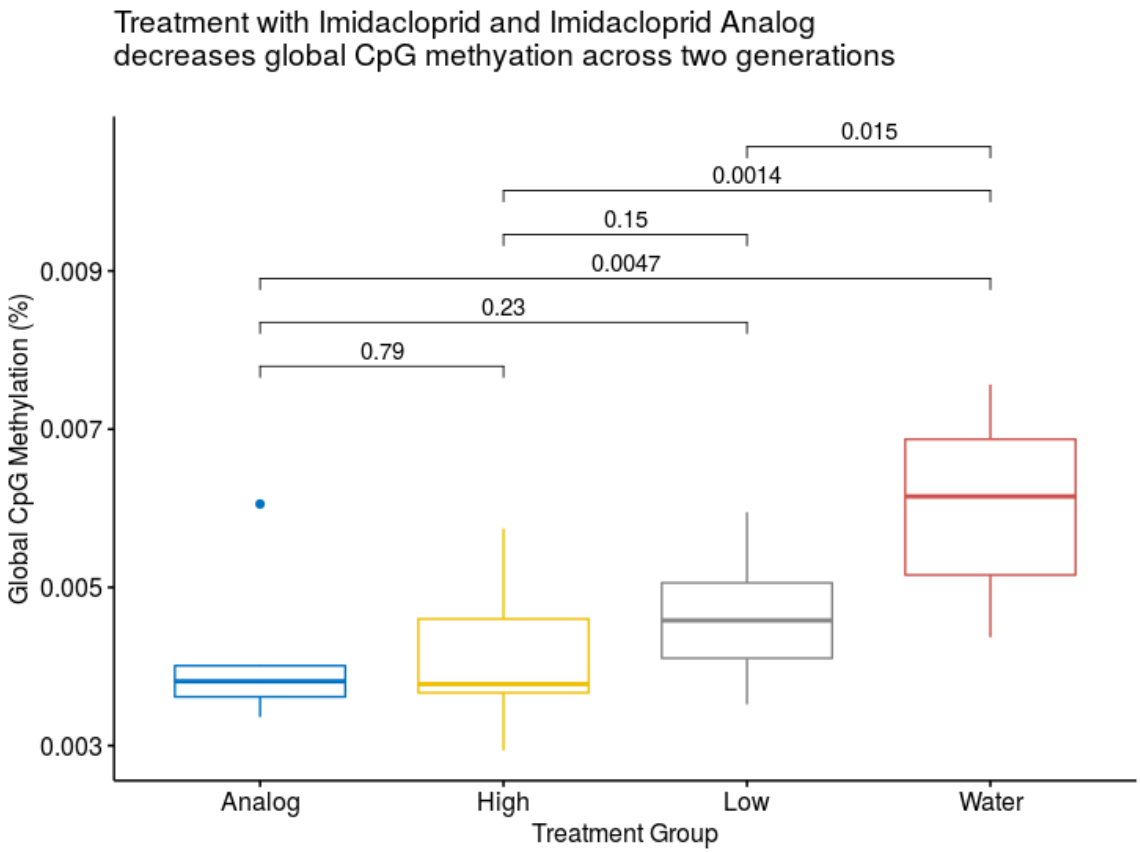

Figure 4.2: ANOVA results of comparison global DNA methylation % of treatment groups with water control. The three treatments (Analog, High imidacloprid, and Low imidacloprid) differ from the water control, but not from each other.

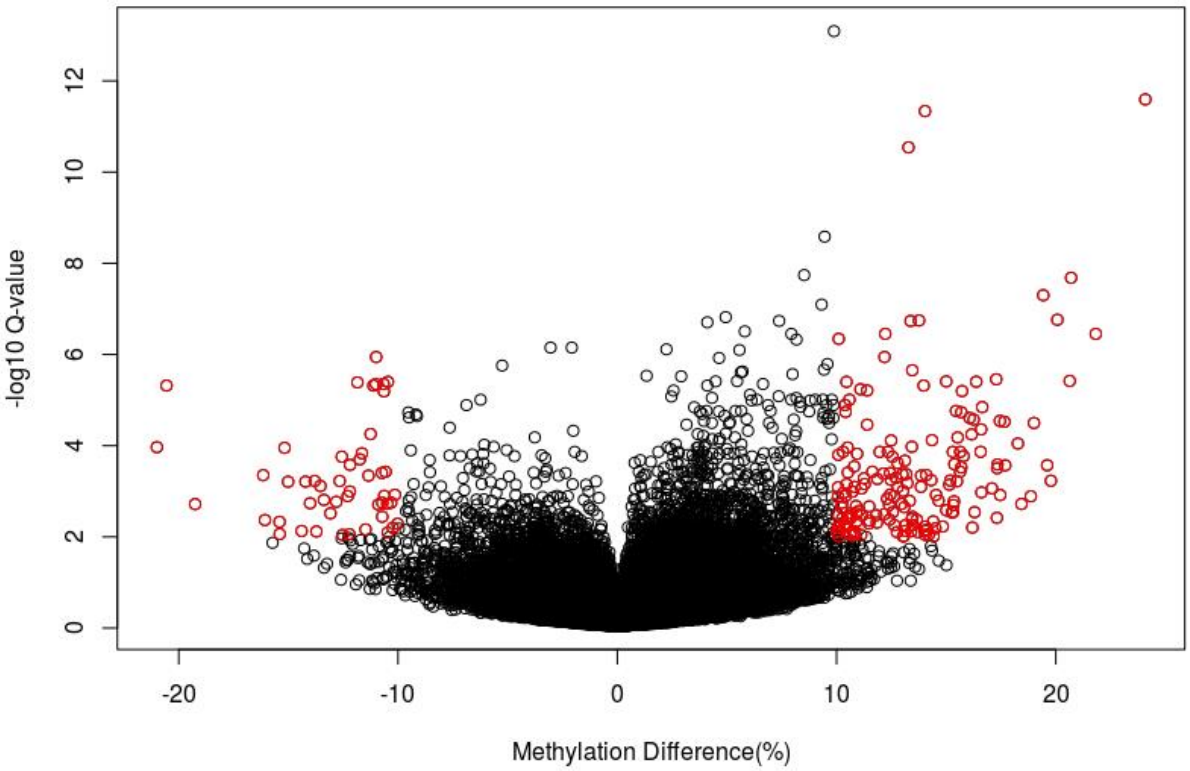

Figure 4.3: Volcano plot showing methylation difference compared to Q-value. Red dots are those selected for further analysis, using a minimum change of 10% methylation level with a Q-value cutoff of 0.01.

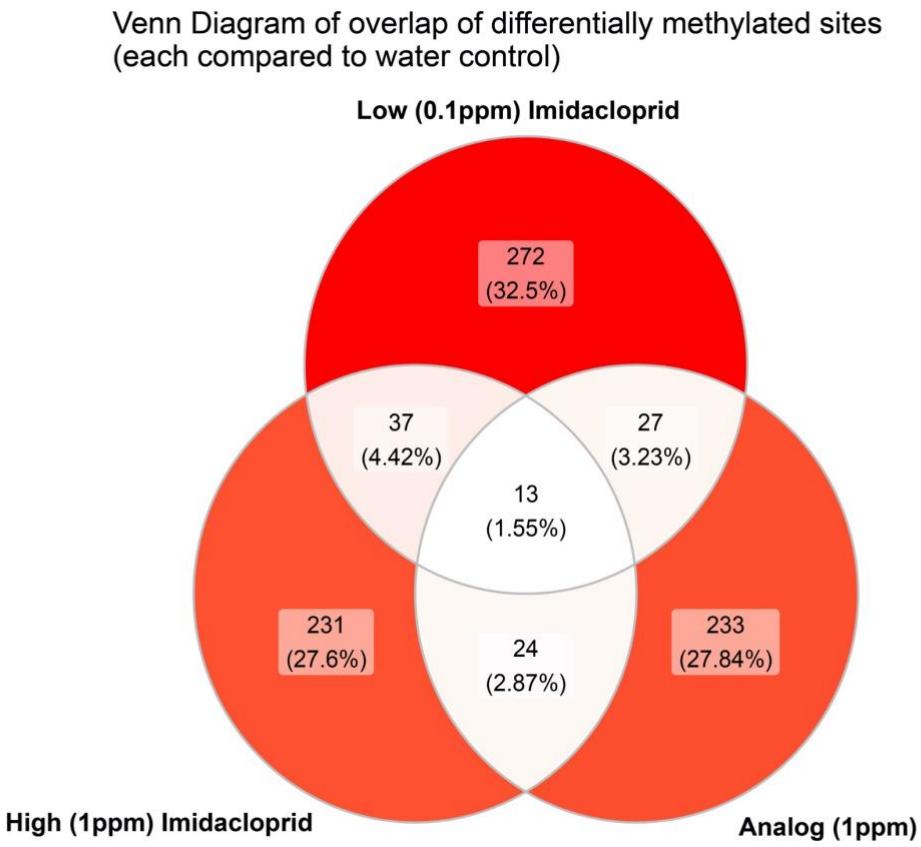

Figure 4.4: Venn diagram illustrating the overlap of differentially methylated sites between treatments (Imidacloprid analog, High imidacloprid, and Low imidacloprid, each compared to a water control.

4.8. Tables

Table 1: Annotated Genes, gene flanking Regions, and transposable Elements that were found to contain differentially methylated sites when all three treatments were together compared to control.

a) Genes within which differentially methylated sites were found			
Gene Name	Gene Function	# of differentially methylated sites	Direction of Change
LDEC011287	Cytochrome P450 (Tribolium castaneum homologue)	3	increased

LDEC015052	Cytochrome P450 (Tribolium castaneum homologue)	2	increased
LDEC004892	Uncharacterized	2	one decreased, one increased
LDEC015089	Putative cyclin-dependent kinase	2	decreased
b) Genes where differentially methylated sites were found within 2kb flanking regions			
Gene Name	Gene Function	# of differentially methylated sites	Direction of Change
LDEC015089	Putative cyclin-dependent kinase	2	one decreased, one increased
LDEC004246	Glycoside hydrolase 45	1	decreased
c) Transposable Elements where differentially methylated sites were found			
Transposable Element Type		# of differentially methylated sites	Direction of Change
LINE/LOA		1	increased
LINE/L2		1	decreased
LINE/Penelope		1	decreased
LINE/Tad1		1	decreased
DNA/PiggyBac		1	decreased
LTR/Gypsy		1	decreased
LINE/L2		5	decreased
DNA/hAT-Charlie		1	decreased
LINE/L2		2	decreased
LINE/Jockey		1	increased
LINE/Jockey		1	increased
LINE/Penelope		1	decreased
DNA/TcMar-Tc1		1	decreased
DNA/PiggyBac		1	decreased
LINE/L2		3	decreased
LINE/CR1		4	decreased
LINE/L2		1	increased
LINE/L2		1	decreased

LINE/L2	3	decreased
LINE/L2	3	decreased
LINE/L2	1	decreased
LINE/L2	1	decreased
LINE/L2	1	decreased
LINE/CR1	2	decreased
LINE/L2	1	increased
LINE/CR1	1	increased
LINE/L2	2	decreased
LTR/Gypsy	1	increased
LINE/L2	1	decreased
LINE/L2	3	decreased
LINE/L2	1	decreased
LINE/L2	1	increased
LINE/L2	1	decreased
DNA/TcMar-Tc1	1	decreased
LTR/Gypsy	2	decreased
LINE/Jockey	1	decreased
LINE/L2	1	decreased
DNA/hAT-Charlie	1	decreased
LINE/Jockey	3	increased
LINE/L2	1	decreased
LINE/L2	1	decreased
DNA/TcMar-Tc1	2	decreased
LINE/L2	1	decreased
LTR/Copia	4	decreased
LTR/Copia	5	decreased
LTR/Copia	4	decreased
LINE/L2	3	decreased

Table 2: Annotated Genes, Gene Flanking Regions, and Transposable Elements which were found to contain differentially methylated sites when each treatment was assessed independently and the results reconciled.

Transposable Elements where differentially methylated sites were found		
Transposable Element Type	# of differentially methylated sites	Direction of Change
LINE/CR1	1	decreased
LINE/L2	1	decreased
LINE/L2	1	decreased

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